

Findings and analysis of novel spreading mode
in influenza virus infection:
Tamiflu-resistant but HA-mediated cell-to-cell
transmission through apical membranes of
cell-associated virions

著者	春山 貴弘
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見と解析:タミフル非感受性 HA 依存型のウイルス粒子非放
出性細胞間伝播)

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筑波大学

春 山 貴 弘

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Findings and analysis of novel spreading mode in influenza virus infection: Tamiflu-resistant but HA-mediated cell-to-cell transmission through apical membranes of cell-associated virions

By

Takahiro Haruyama

Abstract

In first study (Chapter 1), through the evaluation of anti-influenza virus activity which had been exhibited by well-known natural medicine, we serendipitously noticed a strange but an interesting phenomenon in the spreading mode of influenza viruses. In general, it was believed that influenza viruses were released as *cell-free* virions from infected host cells. The released progenitor virions can spread into the outside environment and infect into next host cells far from producer cells. However, our observation suggested that influenza virus had another route for spreading between host cells and could be transmitted to adjacent cells directly without releasing of cell-free virions.

To investigate the possibility, in second study (Chapter 2), we generated release-defect influenza viruses by reverse genetics and performed time-lapse photography. The results clearly showed that influenza virus was capable of spreading via cell-to-cell transmission without enzymatic activities of neuraminidase (NA) which were required for releasing of progenitor virions from infected host cells. Further examination revealed that the cell-to-cell transmission was mediated by mature hemagglutinin (HA) and occurred on the apical surface of polarized host cells. These evidence that cell-to-cell transmission occurs in influenza virus lead to the caution that local infection proceeds even when treated with neuraminidase inhibitors which allow to generate mutant variants. These finding were not only newly but also significant for developing more effective drugs against influenza virus infection.

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Chapter 1

INTRODUCTION

1.1 Influenza virus

Influenza viruses are negative stranded, segmented, enveloped RNA viruses containing helical ribonucleocapsid (also called viral ribonucleoprotein [vRNP]) and belong to the *Orthomyxoviridae* family. There are three types of influenza viruses: A, B and C. Human influenza A and B viruses cause seasonal epidemics of disease almost every year. It causes highly contagious infections of the human respiratory tract [1]. Millions of people are struck by influenza infection each year, resulting in more than 500,000 deaths worldwide. In Japan, the morbidity is almost 10,000 per year and the mortality is calculated as 0.05%. It's causing severe suffering and economic loss. In particular, during a pandemic caused by the emergence of a new and very different influenza virus, the effects are far more deadly (Table 1-1). An estimated 50 million lives were lost in the 1918 "Spanish flu" pandemic, widely regarded as the most devastating pandemic in recorded world history [2]. On the other hands, influenza type C infections cause a mild respiratory illness and are not thought to cause epidemics.

Name of pandemic	Subtype involved	Date	Deaths	Mortality
1918 flu pandemic (Spanish flu) [2]	H1N1	1918–1920	50 million	2%
Asian flu	H2N2	1957–1958	1 to 1.5 million	0.13%
Hong Kong flu	H3N2	1968–1969	0.75 to 1 million	<0.1%
Russian flu	H1N1	1977–1978	no accurate count	N/A
2009 flu pandemic [3]	H1N1	2009–2010	18,000	0.03%

Table 1-1. History of pandemic flu.

Influenza A viruses are classified by antigenic properties of two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Sixteen HA subtypes (H1-H16) and nine NA subtypes (N1-N9) have been defined to the date. Influenza virus infection is limited by the interaction between HA and sialic acid moieties of glycoconjugates on host cells [4]. Viral particles are usually spherical and approximately 100 nm in diameter [5]. The viral envelope consists of lipid bilayer derived from the host plasma membrane but are selectively enriched in cholesterol and glycosphingolipids [6, 7]. The structure of viral envelope can be clearly observed by negative-stained transmission electron micrograph (TEM) (Figure 1-1). The viral envelope contains transmembrane proteins on the outside and matrix protein (M1) on the inside as an undercoat. Three transmembrane envelope proteins; hemagglutinin (HA), neuraminidase (NA) and M2 (ion

channel) are anchored in the lipid bilayer of the viral envelope. HA, a type I transmembrane protein, is a homotrimer and is the major envelope protein (~80%) forming the spikes. HA provides the receptor-binding site and elicits neutralizing antibodies. Cleavage of HA is essential for fusion and virus infectivity. NA, a type II transmembrane protein, is present as a homotetramer on the viral envelope. NA removes the cell surface receptor (sialic acid) and is critical for the release of virus particles from the cell surface and spread of virus. M2, a type III transmembrane protein, is a minor protein component (only 16–20 molecules/virion) of the viral envelope. M2 is a homotetramer, functions as an ion channel [8, 9], and is crucial during uncoating for dissociating the vRNP from M1 in the early phase of the infectious cycle. The viral core consists of helical ribonucleocapsids (vRNP) containing vRNA (negative stranded) and NP and three polymerase proteins (PB1, PB2, PA) which form the viral RNA polymerase complex [10, 11] (Figure 1-2). Influenza A virus has eight gene segments, which encode for the ten proteins (Figure 1-3).

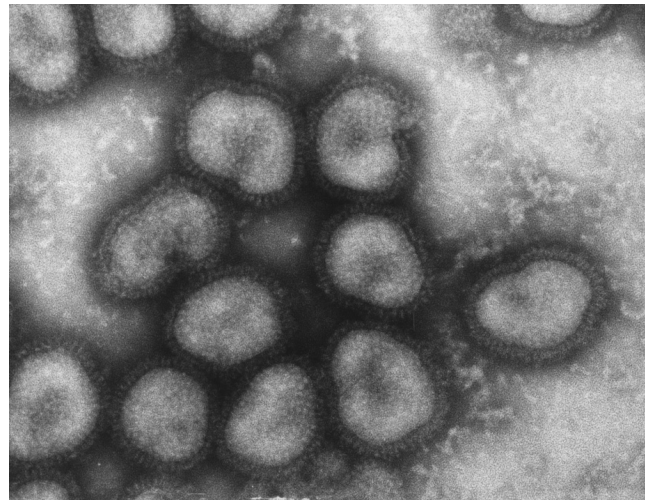


Figure 1-1. Negative-stained transmission electron micrograph (TEM) of influenza virus particles. (virus strain: A/Puerto Rico/8/34)

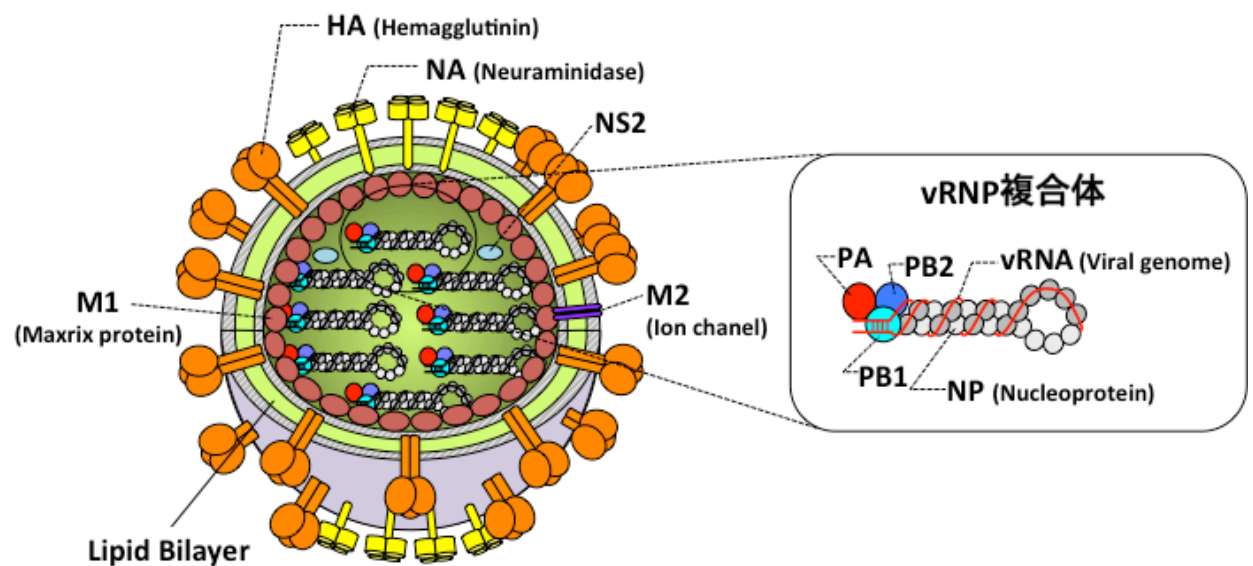


Figure 1-2. Schematic diagram of influenza A virus. Influenza A virus is an enveloped, negative-sense, single-stranded RNA virus. Within the lipid bilayer enveloped, the virus has eight gene segments, which encode for the ten proteins. The viral surface is decorated with spike-like projections of the glycoproteins HA and NA, which are inserted in the lipid bilayer. The viral core consists of helical ribonucleocapsids (i.e. vRNP) containing vRNA (negative stranded) and NP and three polymerase proteins (PB1, PB2, PA) which form the viral RNA polymerase complex.

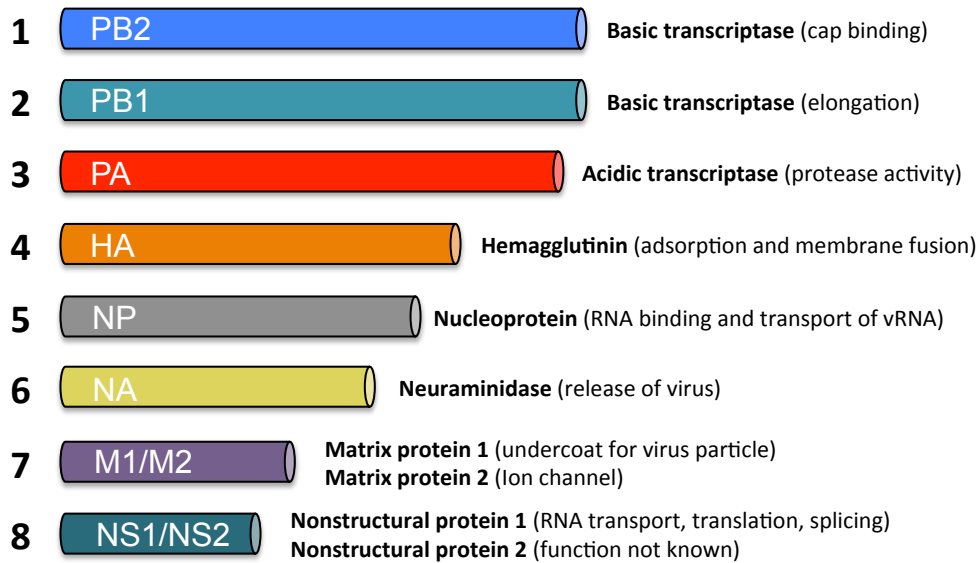


Figure 1-3. Influenzavirus genomes. Influenza A virus has eight gene segments, which encode for the ten proteins. The color bar represents the relative size of vRNA strands. The name of encoded proteins are shown in each bar and the functions of proteins are given in parentheses.

Influenza virus particles bind to cell surface sialic acid, ubiquitously present on glycoproteins or glycolipids. The specificity of the sialic acid (α 2,3-linked or α 2,6-linked sialic acid) and preferred binding of a particular strain of influenza virus to a specific sialic acid receptor are important determinants for species-specific restriction of influenza viruses [12]. During the infectious cycle, virus particles, bound to cell surface sialic acid, are internalized by receptor-mediated endocytosis and viruses possessing cleaved HA undergo fusion with the endosomal membrane [13, 14] at low pH (pH ~5.0). Cleavage of HA is an absolute requirement for infectivity and the nature of the HA cleavage site is an important virulence determinant for influenza viruses. In the acid pH of

the endosome, the cleaved HA undergoes conformational changes releasing the NH₂ terminal fusion peptide of HA2 and causing fusion of viral and endosomal membranes [15].

Virus particles containing uncleaved HA can bind and be endocytosed but cannot undergo fusion and are therefore noninfectious. The M2 ion channel opens up in the acidic pH of the endosome, acidifies the internal virion core, and thereby facilitates the release of vRNP from M1 into the cell cytoplasm. M1-free vRNP is then imported into the nucleus through nuclear pores using nuclear transport signals of NP [16]. Inside the nucleus, vRNP undergoes transcription (mRNA synthesis) and replication (complete positive-sense complementary RNA [cRNA], vRNA [minus strands], and vRNP synthesis) [10, 11].

Progeny vRNPs, made inside the nucleus, are exported out of the nucleus into the cytoplasm with the help of M1 and nuclear export protein (NS2) [17]. Eventually, the envelope proteins (HA, NA, M2), matrix protein (M1) and vRNP (containing vRNA minus-strand, NP, PB2, PB1, PA and NS2) are transported to the assembly site on the plasma membrane where virus particles bud and are released into the outside environment.

NA is required to facilitate the release of newly synthesized viruses from infected cells by cleavage the sialic acid receptor [18, 19]. The life cycle of influenza viruses are summarized in Figure. 1-4.

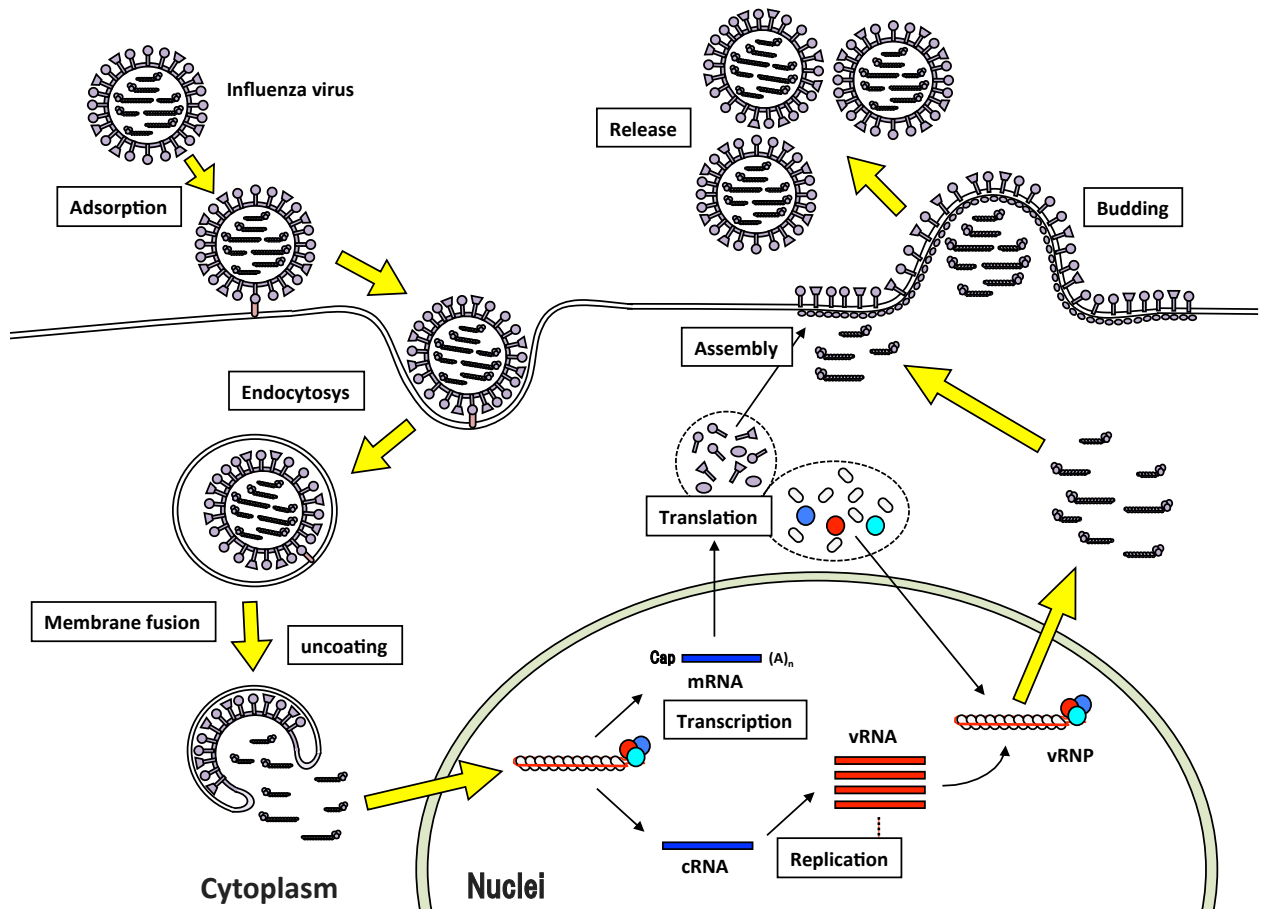


Figure 1-4. Life cycle of influenza virus replication. (i) Adsorption: Influenza viruses attach to host cells via multivalent interactions of HA with sialic acids on cell surface glycoproteins to initiate infection and replication. (ii) Endocytosis: The virus is then internalized by endocytic compartments. (iii) Membrane fusion and uncoating: The H^+ ions enter the virus through the M2 ion channel, and the low pH triggers a conformational change in HA. This activates virus-endosome fusion, and releases the genomic contents into the cytosol. (iv) Intracellular processes: The viral genome is transported into the nucleus, where viral RNA synthesis is carried out by the RNA-dependent RNA polymerase complex, and the viral proteins are expressed through the resulting mRNA. Copies of the negative-strand RNA are also made and packaged into ribonucleoprotein complexes for packaging into new viruses. (v) Assembly, budding and release: The viruses assemble, bud, and are released from the cell membrane. NA cleaves sialic acids from the cell surface proteins to release the virions from the host cell [29].

The widespread 2009 A (H1N1) pandemic, constant antigenic drift of seasonal influenza, and the rapid emergence of antiviral resistance in recent years highlight the ability of this pathogen in adapting to the human population and in evading antiviral drugs [20]. The survival and persistence of influenza virus can be attributed to several of its unique properties. First, the virus transmits easily from person to person by aerosol, and spreads globally through travel and migratory birds. Second, the error-prone viral RNA polymerase lacks proofreading ability, resulting in a high mutation rate of 1.5×10^{-5} per nucleotide per infection cycle [21]. Given the size of the influenza genome of 15,000 bases, this corresponds to an average of one mutation for every 10 viruses produced by an infected cell. This error-prone replication, coupled with the selective pressure of the immune system readily promotes the antigenic variation in the viral proteins HA and NA among seasonal influenza strains [22]. The high mutation rate is also the driving force behind the appearance of drug resistant strains, either in naturally occurring variants, or by drug selection pressure in treated patients [23-25]. Third, the segmented nature of the influenza genome allows for mixing or reassortment of the eight viral gene segments, which can occur in cells infected with two different influenza viruses [26, 27]. The resulting hybrid may contain gene segments from influenza viruses of different species, and can be especially virulent as the population lacks previous exposure to the newly

introduced protein subtypes. Termed antigenic shift, this mechanism underlies the emergence of all three influenza pandemics since the 1918 Spanish flu [28].

1.2 Prophylaxis and Treatment for Influenza Virus

There are currently two distinct strategies in use to control the spread of influenza: vaccines and conventional small-molecule antiviral drugs. Vaccination with the trivalent inactivated or live attenuated vaccines offers only limited protection [29], and is hampered by logistical issues, such as prediction of future circulating strains, and reliability of supply [18]. In the event of a pandemic, rapid production of sufficient quantities of vaccine can be a challenging task [30]. Antivirals present an attractive alternative, and can potentially inhibit viruses of different subtypes or genetic variation. There are six clinically approved antivirals for influenza treatment and prophylaxis (Table 1-2). Amantadine and rimantadine are inhibitors of the M2 ion channel, and interferes with viral fusion [31]. Zanamivir, oseltamivir, peramivir and laninamivir inhibit NA enzymatic activity, thus blocking the release of newly made virions from infected cells [32, 33]. Despite their efficacy, these antivirals suffer from limitations such as a short therapeutic window, high dosage, side effects, and high costs [34-36]. In addition, all the circulating viruses (both

H3N2 and H1N1 strains) are already resistant to the M2 inhibitors [37-39], and resistance to the NA inhibitors has been appearing at an alarming rate in recent years [23, 40].

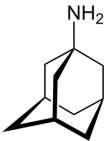
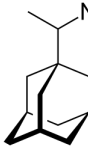
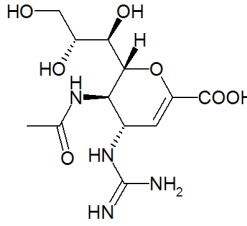
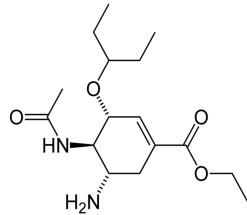
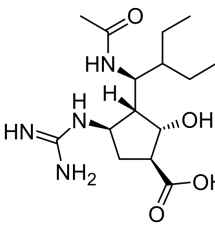
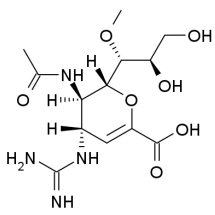
Common name (Trade name) [Manufacture]	Structural formula	Approval in Japan	Administration route
Amantadine (Symmetrel®) [Endo Pharmaceutical]		Approved in 1998	oral drug
Rimantadine (Flumadine®) [Du Pont & Co.]		Not approve	oral drug
Zanamivir (Relenza®) [GlaxoSmithKline]		Approved in 2001	inhalant
Oseltamivir (Tamiflu®) [Roche, Chugai(Japan)]		Approved in 2001	oral drug
Peramivir (Rapiacta®) [Shionogi (Japan), BioCryst Pharmaceuticals]		Approved in 2010	injection
Laninamivir (Inavir®) [Daiichi Sankyo (Japan)]		Approved in 2010	inhalant

Table 1-2. Approved drugs against influenza.

Other influenza antiviral drugs under clinical trials include inhibitors of transcription and genome replication, T-705 (favipiravir) is a pyrazinecarboxamide derivative, and its active form, T-705-4-ribofuranosyl-5'-triphosphate, has been postulated to selectively inhibit the influenza RNA-dependent RNA polymerase [40]. T-705 has been shown to inhibit both oseltamivir- or amantadine-resistant 2009 H1N1 influenza at pM concentrations, however, it is unexpectedly much less effective against dually resistant viruses [41]. In addition, there are few published data on resistance to T-705 [19].

The antiviral drugs under clinical development seem to be promising. Nevertheless, it is expected that resistant strains will eventually develop if these antivirals are used as monotherapy clinically, as that observed with the M2 and NA inhibitors [42]. Therefore, the continuous research and developments of new anti-influenza drugs are both urgent and important. Particular emphasis should be placed on exploring the possibility of combination therapy, and developing novel antivirals with a different mechanism of action that can significantly reduce drug resistance [43, 44].

1.3 Viral spreading mode

Viruses can cross the cellular boundaries and spread from one infected cell to other cells as well as from organism to organism. The mechanism of viral dissemination has been examined in several studies for each virus, since the virus spreading mode is closely related to its pathogenesis and a better understanding of its mechanism can be strongly helpful to develop effective antiviral therapeutic strategies.

It is generally accepted that viruses, released as *cell-free* virions from an infected cell, transmit to distant cells and tissues. This spreading pathway contributes to wide-ranged diffusion of *cell-free* viruses. However, in this spreading pathway, viruses are exposed to host anti-virus defense systems. In contrast, direct infection to a neighboring cell is considered to be beneficial for the virus in terms of evasion from the host anti-virus defense. There are two typical manners in infection to “*right next door*”: One is the virus transmission through cell-cell fusion by forming syncytium without production of progeny virions, and the other is mediated by virions without virus diffusion, generally designated cell-to-cell transmission [45,46].

The cell-cell fusion infection pathway is characteristic for a variety of virus such as paramyxoviruses, herpesviruses, some retroviruses, and so on. For example in the case of measles virus belonging to *Paramyxoviridae*, infection is initiated by the interaction of

the viral hemagglutinin glycoprotein with host cell surface receptors. The virus penetrates into the cell through membrane fusion mediated by the interaction of the fusion glycoprotein. In later stages of infection, newly synthesized glycoproteins accumulate at the cell membrane resulting in fusion of the infected cell with neighboring cells by producing syncytia. Thus, viruses can spread from cell to cell without producing *cell-free* virus particles (Figure 1-5A).

The examples of the cell-to-cell transmission are diverse, and these mechanisms are dependent on pairs of viruses and host cells. Vaccinia virus particles bound on the filopodium of an infected cell are repelled toward neighboring uninfected cells by the formation of filopodia using actin filament [76]. The filopodia direct viruses to uninfected cells. Immunotropic viruses including retroviruses utilize an immunological synapse, designed as virological synapses for the cell-to-cell transmission [52-55]. Claudin-1 and occludin, components of tight junction, are involved in hepatitis C virus (HCV) entry through the cell-to-cell transmission [48,49]. The cell-to-cell transmission through tight junction is also observed in other viruses which infect epithelial layers [50,51]. These retroviruses and HCV remain on the surface of an infected cell even after budding. The uninfected cells adjacent to these infected cells can accept or take over viruses from the infected cell. Thus, the cell-to-cell transmission can be categorized into

two manners based on the state of infecting viruses, either *cell-free* or cell-associated virions (Figure 1-5B).

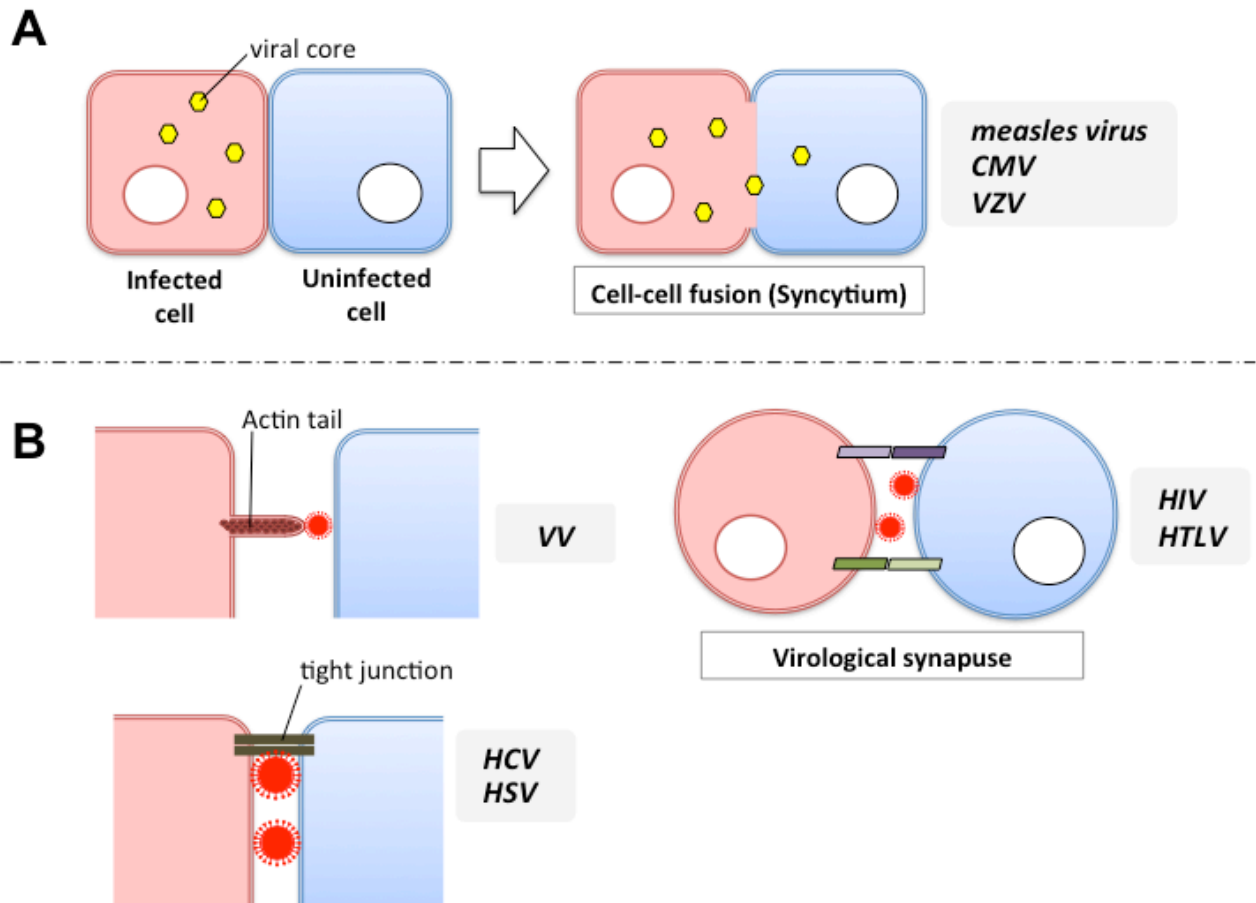


Figure 1-5. Diversity of virus cell-to-cell transmission

(A) cell-to-cell transmission *via* cell-cell fusion. (B) cell-to-cell transmission *via* cell-associated virions. [Abbreviation] CMV: cytomegalovirus, VSV: varicella zoster virus, VV: vaccinia virus, HCV: hepatitis C virus, HSV: herpes simplex virus, HIV: human immunodeficiency virus, HTLV: human T-lymphotrophic virus.

On the other hand, it is generally believed that influenza viruses bud from infected cell surfaces and are efficiently released into the extracellular environment [56. 57]. Since the releasing process is believed as a critical step for the viral transmissibility and

pathogenicity in host organisms, neuraminidase required release of inhibitors the target of antiviral therapeutic have tended to be focus on the prevention of the virus release in recent years. Although influenza viruses also belong to enveloped viruses, the existence of cell-to-cell transmission and its contribution have been hardly described so far.

1.4 Motivation for this thesis and thesis outline

Recently, several kinds of air cleaner are developed and used for the elimination of dust or toxic substances from air. The pathogens related to respiratory infectious disease are also one of the important targets for such cleaners. This study had started from the investigation about a material coated on the filter unit of an air cleaner. We tested whether the material can eliminate influenza viruses from air by trapping the virions on it. The material was one of famous medical plant: *Ginkgo biloba* leaf extract (EGb). Through the investigation, EGb was not only capable of trapping the virions but also markedly reducing the infectivity of influenza viruses. Since EGb was hardly described as an antiviral substance so far, we focused on the anti-influenza virus activity of EGb itself.

In first study (Chapter 2), we evaluated anti-influenza virus activities of EGb to find new candidate for the influenza therapeutic. Through the evaluation, we serendipitously noticed the strange but interesting phenomena in the spreading mode of influenza viruses. In general, it was believed that influenza viruses were released as *cell-free* virions from infected host cells. Therefore, progenitor virions can spread into the outside environment and infect into next host cells far from producer cells. This property is useful to explain about the extremely high transmission ability of influenza viruses. However, our

observation in this study could not be explained by such conventional concepts and suggested the existence of unknown nature of influenza viruses in spreading between cells. This finding became the strong motivation to investigate the existence of novel transmission modes in influenza virus infection.

In second study (Chapter 3), we focused on the transmission mode of influenza viruses. By reverse genetics, we generated recombinant influenza viruses that expressed GFP instead of NA and performed time-lapse photography *in vitro*. These results suggested that influenza viruses were capable of spreading via cell-to-cell transmission mode independent on NA activities. Furthermore, we examined the details of the newly transmission mode of influenza viruses and its significances.

In final section (Chapter 4), I summarized our findings and discussed about the significances of cell-to-cell transmission in influenza virus infection. Furthermore, I proposed future directions of this study, so that further research about novel transmission modes of influenza viruses may lead to new insights and concepts for developing more effective drugs.

Chapter 2

ANTI-INFLUENZA VIRUS ACTIVITY OF GINKGO BILOBA LEAF EXTRACTS

Abstract

We have examined the influence of *Ginkgo biloba* leaf extract (EGb) on the infectivity of influenza viruses in Madin-Darby canine kidney (MDCK) cells. Plaque formation assays demonstrated that the multiplication of influenza viruses after the viral adsorption onto host cells was not affected in the overlaid agarose gel containing EGb. However, when influenza viruses were pre-treated with EGb prior to exposure to cells, their infectivity was markedly reduced. In contrast, the inhibitory effect was not observed when MDCK cells were pre-treated with EGb before the infection of influenza viruses. Hemagglutination inhibition assays revealed that EGb interferes with the interaction between influenza viruses and erythrocytes. The antiviral effect of EGb was observed against influenza A (H1N1), and A (H3N2), and influenza B viruses. These results suggested that EGb contains an anti-influenza virus substance(s), which directly affects influenza virus particles and disrupts the function of viral hemagglutinin for virus adsorption to host cells. In addition to the finding of the anti-influenza virus activity of

EGb, our results demonstrated interesting and important insights into the screening system for anti-influenza virus activities. In general, the plaque formation assay by using overlaid agarose gel containing drugs is used as the most authentic method to detect antiviral activities. However, our results showed that EGb had no effects on not only the number of plaques but also the size of them in the plaque formation assay. These may suggest the existence of “overlooked” inhibitory activities against influenza virus in past studies.

Introduction

Influenza viruses, belonging to the family of Orthomyxoviridae, cause the epidemic disease in the human population every year despite the availability of effective vaccines. In a severe pandemic year, millions of people die from the virus infection. Influenza viruses are classified by antigenic properties of two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Sixteen HA subtypes (H1-H16) and nine NA subtypes (N1-N9) have been defined to date. Influenza virus infection is limited by the interaction between HA and sialic acid moieties of glycoconjugates on host cells [4].

Some synthetic drugs such as amantadine and remantadine (both of them are known as the inhibitor of M2 ion channel), oseltamivir and zanamivir (both of them are known as neuraminidase inhibitor) have been available for decades, but all of them have side effects and some limitation in use [60, 64]. Therefore, novel substances and approaches are needed to control and prevent the virus disease. The various natural products have distinct anti-influenza virus activities [67]. We have demonstrated that the high molecular weight lignin-related fraction from pine cone extracts (PCE) of *Pinus parviflora* Sieb. et Zucc. suppresses the multiplication of influenza virus through prevention of the viral RNA synthesis [62, 68]. We reported that *Sanicula europaea* L. leaf extracts contain an anti-influenza virus substance(s), which selectively inhibits influenza A virus,

but not influenza B virus [66]. Studies on natural products in terms of their anti-influenza virus activity have increased dramatically in the past several years [67].

Ginkgo biloba leaf extract (EGb) is known as a potential phytomedicine having various drug actions, in particular, anti-coagulant, vasodilator, and anti-inflammatory activities [69]. In many countries, EGb and similar products have been prescribed as therapeutic medicines for cerebral and peripheral vascular inefficiency and cognitive impairments associated with aging [58, 59]. Unlike other herbal drugs, EGb has hardly been tested in its anti-influenza virus activity. In the present study, we have examined the antiviral effect of EGb on influenza viruses.

Results

Effect of EGb on the viability and growth of MDCK cells

Prior to the examination of the anti-influenza virus activity of EGb, we investigated whether EGb affects the viability and growth of MDCK cells, host cells routinely used for influenza viruses. The cell viability and growth were evaluated by counting the number of living cells as a function of time using the neutral red assay as described in Materials and Methods. The cytotoxic effects of EGb were not observed at the concentrations less than 10 µg/ml ($CC_{50} = 180$ µg/ml) (Figure 2-1A). Neither the growth rate nor the final cell density were affected in the presence of 10 µg/ml of EGb (Figure 2-1B), while a marked decrease in the cell growth rate was observed at 100 µg/ml (Figure 2-1B). Thus, EGb at the concentration less than 10 µg/ml could be considered essentially non-toxic to MDCK cells. It was confirmed that the solvent DMSO had no effect on the viability and the growth of MDCK cells in the range of concentrations used in this study (data not shown).

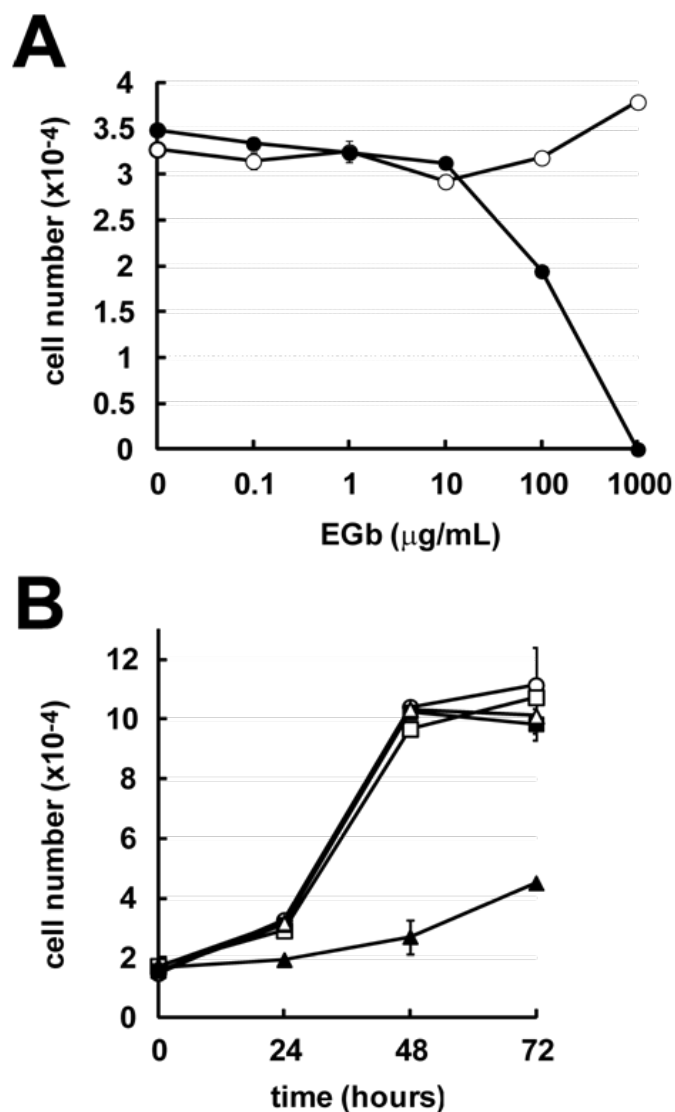


Figure 2-1 Effect of EGb on the viability and the growth of MDCK cells. (A) MDCK cells (3.5×10^4) were seeded in 24-well tissue culture plates and incubated at 37°C in the presence of various concentrations of EGb (closed circles) or solvent DMSO alone (open circles). After incubation for 24 h, the viable cell number was determined by the neutral red assay. (B) MDCK cells (2×10^4) were seeded in 24-well tissue culture plates and incubated at 37°C in the absence (open circles) or presence of 10 µg/ml (closed square) and 100 µg/ml (closed triangles) of EGb, and 0.01%(v/v) and 0.1%(v/v) of DMSO alone (open square and open triangle, respectively). After incubation for the indicated periods, the viable cell number was determined by the neutral red assay.

Inhibition of the influenza virus infectivity by EGb

To examine whether EGb is inhibitory for multiplication of influenza virus, plaque assays were carried out as described in Materials and Methods. Cells were infected with influenza A/PR/8/34 virus at 37°C for 1 h. The cells were washed extensively with serum-free MEM and then overlaid with the overlay medium (0.8% agarose in MEM) containing EGb at various concentrations. The number of plaques and the size of them in the presence of EGb were not different from those in the absence of EGb (Figure. 2-2A), indicating that EGb does not inhibit the plaque formation by influenza virus infection. We further examined whether EGb is effective when mixed with viruses prior to exposure to cells. Influenza virus was mixed with EGb at various concentrations at room temperature for 10 minutes and then exposed to MDCK cells. In this case, EGb markedly inhibited the virus infectivity in a dose-dependent manner (Figure 2-2B). EGb at the concentration of 5 µg/ml inhibited almost completely the plaque forming activity ($IC_{50} = 1.86 \mu\text{g/ml}$). These suggest that EGb inhibits the initial step of influenza virus infection prior to the viral internalization into the cytoplasm. Next, we examined whether the inhibitory effect of EGb was directly or indirectly against influenza virus. Plaque formation assays were performed using MDCK cells which were pre-treated with EGb at various concentrations for 1 h before the inoculation of influenza viruses. There was no

statistically significant difference in the number of plaques and their sizes between tested groups in the presence of EGb and the control group in the absence of EGb (Figure 2-3).

It was suggested that EGb directly interacts with influenza viruses and markedly reduced the infectivity.

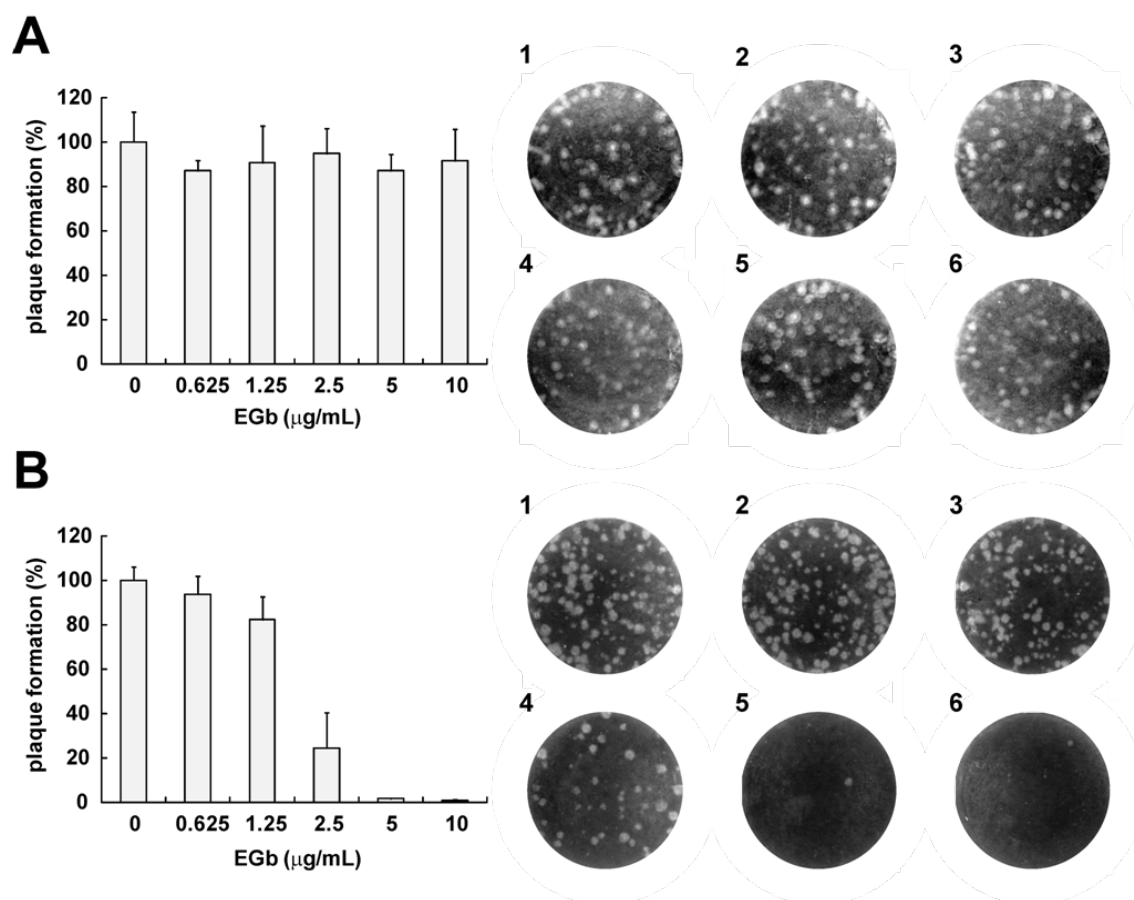


Figure 2-2 Effect of EGb on plaque formation. Plaque assays were carried out as described in Materials and Methods. (A) MDCK cells were infected with virus suspension (500 pfu/ml) and then overlaid with the overlay medium containing various concentrations of EGb. The profile of plaques was shown in right panels. Panels: 1, 2, 3, 4, 5, and 6 represent assays carried out in the presence of 0, 0.625, 1.25, 2.5, 5, and 10 $\mu\text{g/ml}$ of EGb, respectively. (B) Influenza A virus (500 pfu/ml) was incubated with various concentrations of EGb prior to exposure to MDCK cells. The profile of plaques was shown in right panels. Panels: 1, 2, 3, 4, 5, and 6 represent assays in the presence of 0, 0.625, 1.25, 2.5, 5, and 10 $\mu\text{g/ml}$ of EGb, respectively. Results are represented as the value relative to the percent of the plaque number formed in the absence of EGb.

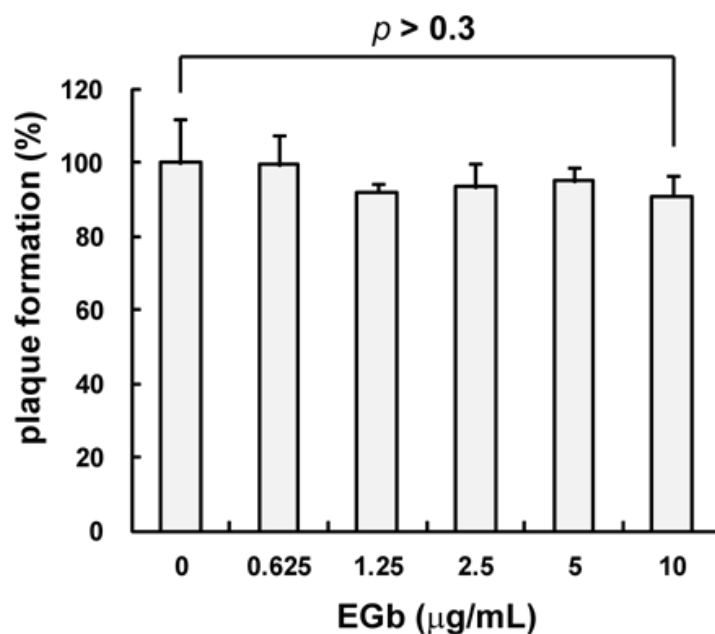


Figure 2-3 Effect of pre-treatment of host cells with EGb on influenza virus infection. MDCK cells were exposed by EGb at various concentrations and incubated at 37°C for 1h prior to virus infections. After removing EGb, MDCK cells were inoculated with influenza A/PR/8/34 viruses (500 pfu/ml), and plaque formation assays were carried out as described Materials and Methods. Results are represented as the value relative to the percent of the plaque number formed in the absence of EGb. All data were represented as mean \pm SD, and the statistical analysis was performed using t-test to compare two groups.

Inhibition of hemagglutination by EGb

Influenza virus infection initiates with the interaction of hemagglutinin (HA) on the virion with sialic acids on the cell surface. To understand how EGb prevents the virus adsorption to cells, we examined whether EGb inhibits the influenza virus-mediated hemagglutination competitively. As shown in Figure. 2-4, EGb inhibited hemagglutination in a dose-dependent manner, suggesting that EGb interferes with the interaction between HA and sialic acids.

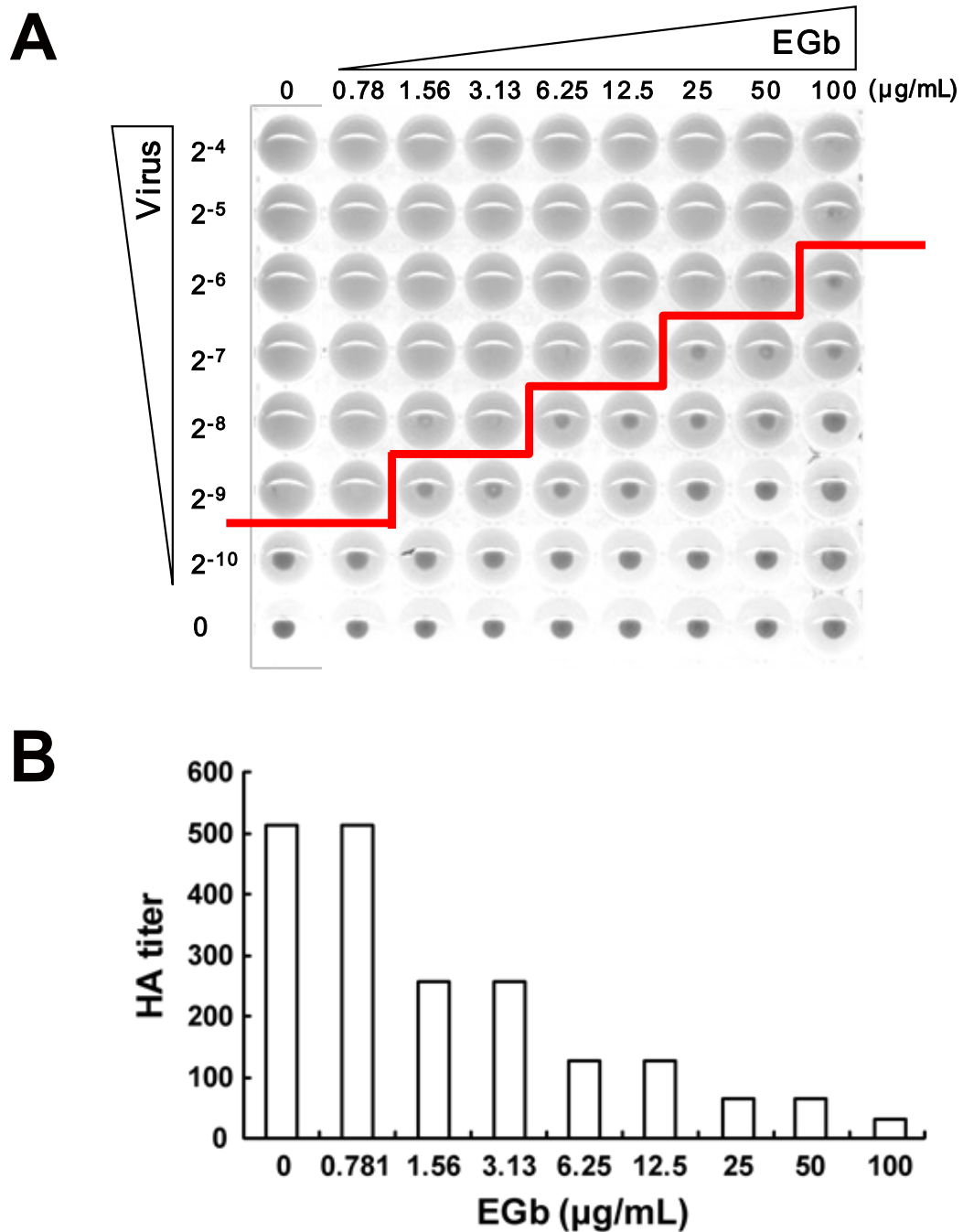


Figure 2-4 HA titers of influenza A virus treated with various concentrations of EGb. (A) Influenza A/PR/8/34 virus and EGb were diluted by 2-fold dilution at each time and then mixed. After incubation at room temperature for 5 min, 0.5% chicken erythrocyte suspension was added to each of these mixtures in a 96-well assay plate, and the plate was incubated at room temperature for 30 min for hemagglutination. (B) Results were represented as a plot where the x-axis and y-axis indicate concentrations of EGb and HA titer, respectively. The result is representative of three independent experiments.

Susceptibility of other influenza virus strains to EGb

Our results suggest that EGb binds to HA and prevents the virus adsorption to cells. We further examined whether the inhibitory effect of EGb is dependent on the type of influenza viruses. EGb inhibited the infectivity of both A/Udorn/72 (H3N2) and B/Lee/40 viruses as well as A/PR/8/34 (H1N1) in an adsorption inhibition-dependent manner (compare Figure 2-5A and 2-5B), although the sensitivity was slightly different. The 50% inhibitory concentration (IC_{50}) value of EGb was calculated for three different types of influenza viruses which examined in this study. Furthermore, the selectivity index (SI) was evaluated as the ratio of CC_{50} to IC_{50} and represented in Table 2-1. Influenza A/PR/8/34 virus was the most sensitive to EGb (Table 2-1). These suggest that the anti-viral activity of EGb is not dependent on the types of influenza viruses.

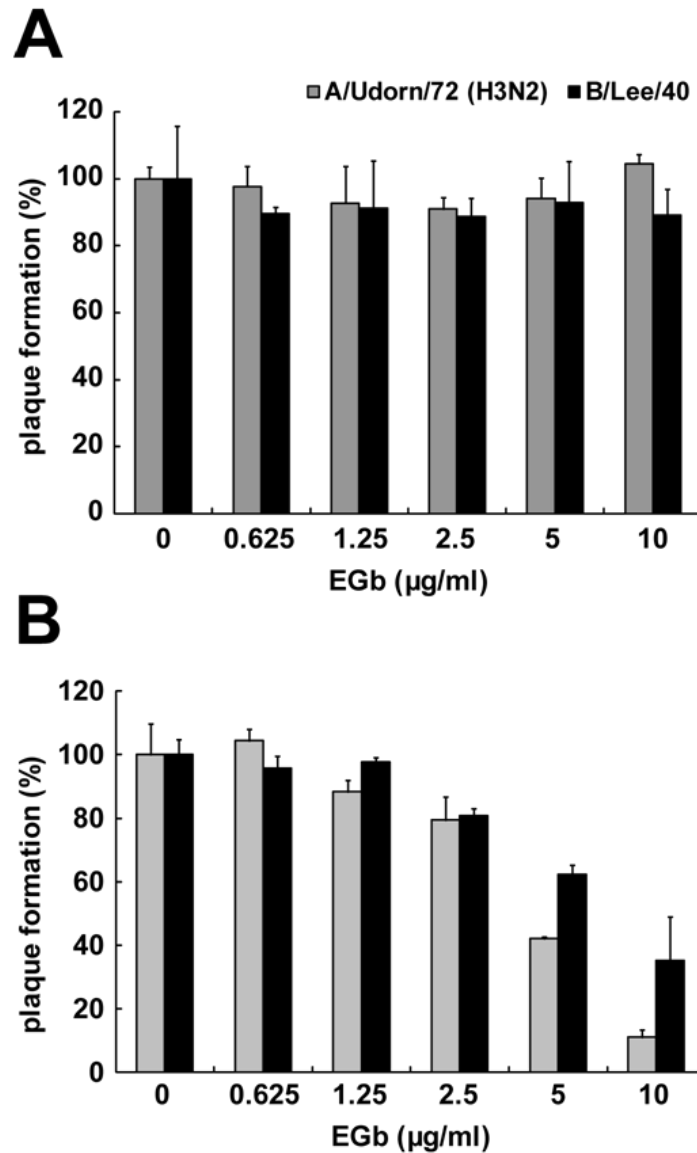


Figure 2-5 Effect of EGb on plaque formation by two different subtypes of influenza virus. Plaque assays were carried out as described Materials and Methods. **(A)** MDCK cells were infected with 0.5 ml of 500 pfu/ml of influenza A/Udorn/72 (H3N2), and B/Lee/40 viruses and then overlaid with the overlay medium containing various concentrations of EGb. **(B)** Each influenza virus strain was diluted to 500 pfu/ml and incubated with various concentrations of EGb prior to exposure to MDCK cells. One hour after virus inoculation, MDCK cells were washed with serum-free MEM and subsequently overlaid with the overlay medium without EGb. Results are represented as the percent of the plaque number formed in the absence of EGb. In Fig. 2-5A and 5B, results of A/Udorn/72 (H3N2) and B/Lee/40 are represented by gray bar and black bar, respectively.

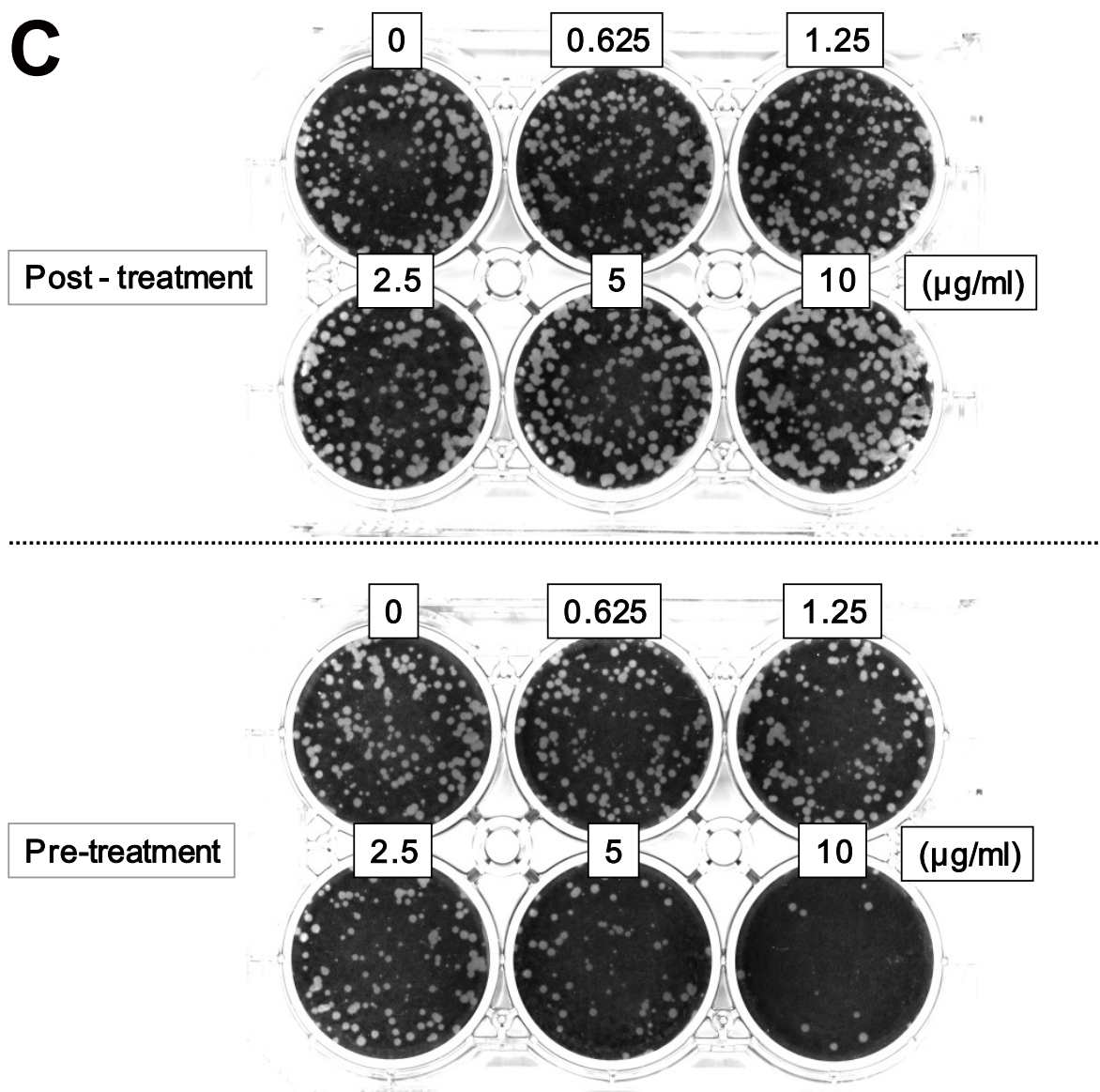


Figure 2-5 (C) Results of plaque assay for A/Udorn/72 (H3N2) was represented.

virus strain	IC₅₀ (µg/ml)^a	SI^b
A/PR/8/34 (H1N1)	1.86	96.8
A/Udorn/72 (H3N2)	4.41	40.8
B/Lee/40	6.79	26.5

Table 2-1 Selectivity indices of EGb in three different influenza virus strains.

^a IC₅₀: 50% Inhibitory Concentration of EGb was calculated from the results of the plaque formation assay performed as shown in Fig. 2-2b and Fig. 2-5b. ^b SI: Selectivity Index was evaluated as the ratio of CC₅₀ to IC₅₀, *i.e.*, $SI = CC_{50}/IC_{50}$. CC₅₀: 50% Cytotoxic Concentration of EGb was calculated from the dose-response curve shown in Fig. 2-1a and its value (= 180 µg/mL) was used for the calculation of each SI. All calculation was performed by using GraphPad Prism software as described in Materials and Methods.

Discussion

In this study, we found the anti-influenza virus activity in *Ginkgo biloba* leaf extract (EGb). Our results showed that EGb acted directly on influenza viruses and prevented the viral adsorption onto the host cell surface, suggesting that EGb interfered the interaction between influenza virus HAs and sialic acids on the host cell surface, although we could not exclude the possibility that EGb had a virucidal activity and directly inactivated influenza virus (Figure 2-6).

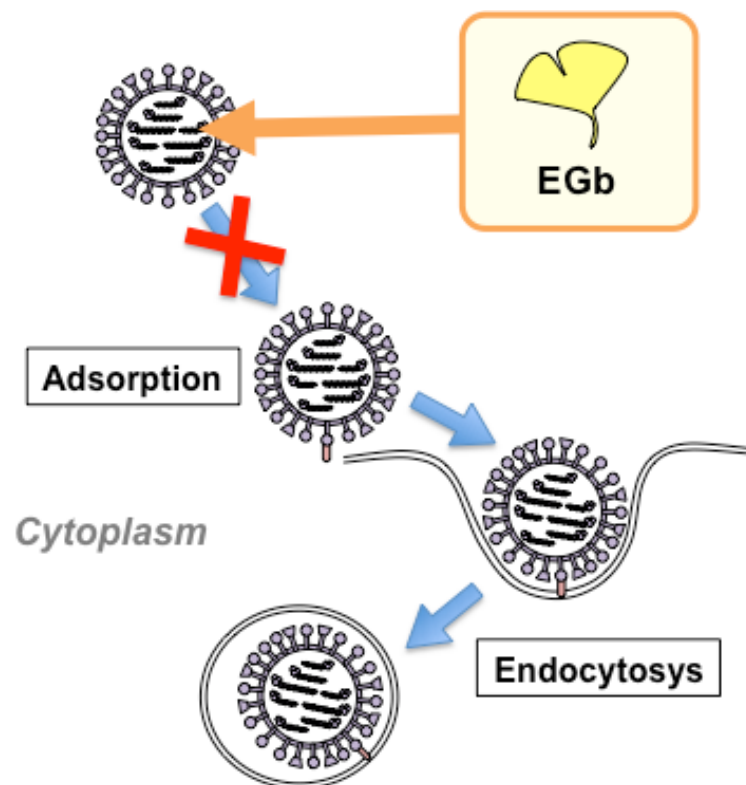


Figure 2-6. Working hypothesis of EGb to prevent influenza virus infection.

The contents of an active constituent(s) in EGb are standardized around the world, *i.e.*, 24% of ginkgo flavonol glycosides (quercetin, kaempferol, and isorhammetin) and 6% of terpene lactones (ginkgolides and bilobalide). EGb also contains a class of condensed tannins, which are polymers of primarily flavan-3-ols (catechin and epicatechin) with a covalent bond between the individual flavonol units. Nakayama et al. have previously reported that tea-condensed tannins, (-)epigallocatechin gallate (EGCG) and teaflavin digallate, bind to the haemagglutinin of influenza virus, and inhibit its adsorption to MDCK cells [63]. Furthermore, Song et al. have shown that catechin-derivatives, including EGCG from green tea, inhibit not only the influenza virus hemagglutination but also the viral neuraminidase activity [65], the latter of which is thought to play a key role in the release of progeny virions from infected cells by cleavage of sialic acid moieties of host cell receptors and the prevention of self-aggregation of virions by cleavage of sialic acid still bound to the virus surface. These findings provide important insights into the molecular mechanism of the action of EGb.

Ginkgetin is a biflavone originally isolated from *Ginkgo biloba* leaf and has been found to inhibit the influenza virus sialidase [61]. However, our results showed that EGb prevented the viral adsorption in the initial step of influenza virus infection. Therefore, in our case, an effective substance(s) in EGb may be different from Ginkgetin.

EGb was effective on three different types of influenza viruses tested so far, *i.e.*, influenza A/PR/8/34 (H1N1), A/Udorn/72 (H3N2), and B/Lee/40 viruses, even though the sensitivity towards EGb was slightly different among them. It is suggested that EGb may be potential have wide range inhibitor against influenza virus infection.

As shown in Figure 2-2A, when plaque formation assays were performed with overlay medium containing EGb, there were no effects on not only the number of plaques but also their sizes. Since our results suggested that EGb acts directly on influenza virus and prevent the initial step of viral infections, it was expected that the infectivity of progenitor virions would be decreased due to interaction with EGb present in overlay medium and consequently the size of an individual plaque should be reduced under the plaque formation assay. The discrepancy between the predicted results and experimental results may raise an interesting and newly insight into influenza virus spreading modes. In general, it has been believed that influenza viruses were capable of spreading via *cell-free* virions released from infected cells depending on the enzymatic activity of NA. In plaque assay, if *cell-free* virions were released into overlay medium containing agarose and antiviral agents (*i.e.*, EGb) at active concentrations, the *cell-free* virions might have the opportunity to interact with EGb in microenvironments and its infectivity was decreased

immediately as well as in case of pre-treatment experiments. However there was no changing in the plaque size according to actual results.

The initial step of virus infection has been generally described as the binding of *cell-free* virions to their host cell surface followed by internalization and replication. However, some kinds of virus, even if the virus does not have activities to form syncytium, also spread between cells without diffusing of progenitor virions into the extracellular environment. Recently, it has been highlighted the significance of such a secondary viral spreading mode [45, 46]. The secondary mode is often designated cell-to-cell transmission [45]. In the cell-to-cell transmission, progenitor virions are remaining on the surface of the producer cell even after budding, and cross the cellular boundaries via the cell-cell contact. Therefore, newly produced virions are capable of spreading between adjacent cells, directly. For instance, Hepatitis C virus (HCV) is able to switch the spreading mode from via *cell-free* virions to cell-to-cell transmission so that progenitor virions can be escaped from host immune defence. Claudin-1 and occludin known as components of tight junction are involved in HCV entry to target host cells through cell-to-cell transmission [48, 49]. Besides the case of HCV infection, the cell-to-cell transmission had been also observed in other enveloped viruses (HSV-1[70-72], HTLV-1[54], HIV-1[73-75] and so on). Thus, the cell-to-cell transmission certainly plays

significant roles for the dissemination of several enveloped viruses. The strange results in this study raise one possibility that influenza viruses can spread without diffusing of progenitor *cell-free* virions and it may be capable of transferring to adjacent cells via cell-to-cell transmission as well as HCV.

In conclusion, the results presented here showed that EGb interacts directly with influenza viruses and markedly reduces the infectivity by preventing the virus adsorption to host cells. Furthermore, it was suggested that the inhibitory effect of EGb was not restricted to a certain subtype of influenza viruses. Taken together, EGb is considered to be useful as a prevention agent against influenza virus infection, although further studies are necessary to confirm the anti-influenza virus activity *in vivo*.

In addition to the finding of the anti-influenza virus activity of EGb, we demonstrated an interesting and important insight(s) into the screening system for the anti-influenza virus activity. As is the case for the anti-influenza virus activity of EGb found in this study, some candidates for antiviral agents had been overlooked in past studies because of the existence of unknown transmission mode of influenza viruses. Our results raise a caution for investigators who try to find anti-influenza virus compounds.

In next study (chapter 3), we went to investigate the possibility that influenza viruses were capable of spreading via cell-to-cell transmission without *cell-free* virion.

Materials and Methods

Reagents

The powder of *Ginkgo biloba* leaf extract was gifted from Mitsubishi Paper Mills Co., Ltd., Japan, and dissolved in DMSO at the concentration of 100 mg/ml and stored at –30°C until use.

Viruses

Influenza A/PR/8/34 (H1N1), A/Udorn/72 (H3N2), and B/Lee/40 viruses were grown at 35.5°C for 48 h in allantoic sacs of 11 days old embryonated eggs (MIYAKE HATCHERY), and then the infected allantoic fluid was collected and stored at –80°C until use.

Cells

Madin-Darby canine kidney (MDCK) cells were maintained in Eagle's minimum essential medium (MEM) at 37°C, in 5% CO₂ atmosphere, supplemented with 10% fetal bovine serum, 0.03% L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Neutral red assay

The neutral red assay is based on incorporation of neutral red into lysosomes in living cells. To determine the effect of EGb on the cell viability, MDCK cells (3.5×10^4 cells/well) were seeded into 24-well tissue culture plates and kept at 37°C overnight. After removal of the culture medium, 0.4 ml of MEM containing various concentrations of EGb or DMSO was added to each well of the plates. After incubation for 24 h at 37°C, 0.2 ml of neutral red solution (0.15 mg/ml) was added to each well. After incubation at 37°C for 3h, wells were washed with 0.2 ml of a fixative (1% formalin and 1% CaCl_2). To extract the dye, 0.2 ml of 1% acetic acid in 50% ethanol was added to each well. After incubation at room temperature for 20 min, the amount of neutral red in each well was determined by measuring absorbance at 550 nm using a spectrometer. Results were represented as the cell number that was calculated from the standard curve of cell numbers. Furthermore, to determine the effect of EGb on the cell growth, MDCK cells (2.0×10^4 cells/well) were seeded into 24-well tissue culture plates and kept at 37°C overnight. After removal of the medium, 0.4 ml of MEM containing 0, 10, and 100 $\mu\text{g/ml}$ of EGb were added to each well. As control groups, DMSO was added to each well at final concentrations of 0.01% or 0.1%. After incubation at 37°C for 0, 24, 48, and 72 h, viable cells were determined with the neutral red assay as described above.

Treatment of viruses and cells by EGb

For pre-treatment of viruses by EGb, influenza A/PR/8/34 virus (500 pfu/ml) was mixed with EGb at several concentrations, incubated at room temperature for 10 minutes, and then subjected to plaque formation assay. For post-treatment by EGb, MDCK cells infected with influenza viruses were overlaid with 0.8% agarose containing EGb at several concentrations in plaque formation assay. To investigate the direct effect of EGb on host cells, MDCK cells were exposed by EGb at several concentrations and incubated at 37°C for 1 h. After removing the medium containing EGb, MDCK cells were infected with influenza viruses followed by the plaque formation assay.

Plaque formation assay

A confluent monolayer culture of MDCK cells in a 6-well tissue culture plates was washed with serum-free MEM and then infected with 0.5 ml of influenza virus solution (500 pfu/ml = MOI of 2.5×10^{-4}) in serum-free MEM. After allowing at 37°C for 1 h for virus adsorption, the cells were washed with serum-free MEM and then overlaid with MEM containing 0.8% agarose, 0.2% BSA, and 1 µg/ml TPCK (L-1-Tosylamide-2-phenylethyl chloromethyl ketone) treated trypsin (sigma). After

incubation at 37°C for 2 - 3 days, plaques were visualized by staining cells with 0.5% amido black. Results were represented as a ratio of the plaque number formed in the presence of EGb to that in the absence of EGb.

Hemagglutination assay

Influenza A/PR/8/34 virus (2×10^8 pfu/ml) was diluted 9 times with PBS (-) by 2-fold dilution at each time, while 200 µg/ml of EGb were also diluted 10 times with PBS(-) containing 0.2% DMSO by 2-fold dilution at each time. Fifty microliter of each diluted virus was mixed with 50 µl of each diluted EGb. These mixtures were then maintained at room temperature for 5 min. One hundred microliter of 0.5% chicken erythrocyte suspension (Nippon Bio-Test Laboratories Inc., Japan) was added to each of these mixtures in 96-well round bottom plates, and then the plate was incubated at room temperature for 30 min for hemagglutination. Results were represented as a plot where the x-axis and y-axis indicate concentrations of EGb and HA titer, respectively.

Statistical analysis

All of the data were represented as mean \pm standard error of the mean (SEM). Comparisons for all pairs were performed by the Student *t*-test. A *p* value > 0.05 was

considered to be no significant. The calculation of 50% cytotoxicity concentration (CC_{50}) and effective concentrations with 50% plaque reduction (EC_{50}) were performed by nonlinear regression using GraphPad Prism's "log (inhibitor) vs. response – variable slope" function (GraphPad Prism Version 5.01 for Windows, GraphPad Software Inc.).

Chapter 3

TAMIFLU[®]-RESISTANT BUT HA-MEDIATED CELL-TO-CELL TRANSMISSION THROUGH APICAL MEMBRANES OF CELL-ASSOCIATED INFLUENZA VIRUSES

Abstract

The infection of viruses to a neighboring cell is considered to be beneficial in terms of evasion from host anti-virus defense systems. There are two pathways for viral infection to “*right next door*”: One is the virus transmission through cell-cell fusion by forming syncytium without production of progeny virions, and the other is mediated by virions without virus diffusion, generally designated cell-to-cell transmission. Influenza viruses are believed to be transmitted as *cell-free* virus from infected cells to uninfected cells. Here, we demonstrated that influenza virus can utilize cell-to-cell transmission pathway through apical membranes, by handover of virions on the surface of an infected cell to adjacent host cells. Live cell imaging techniques showed that a recombinant influenza virus, in which the *neuraminidase* gene was replaced with the *green fluorescence protein* gene, spreads from an infected cell to adjacent cells forming infected cell clusters.

This type of virus spreading requires HA activation by protease treatment. The cell-to-cell transmission was also blocked by amantadine, which inhibits the acidification of endosomes required for uncoating of influenza virus particles in endosomes, indicating that functional hemagglutinin and endosome acidification by M2 ion channel were essential for the cell-to-cell influenza virus transmission. Furthermore, in the cell-to-cell transmission of influenza virus, progeny virions could remain associated with the surface of infected cell even after budding, for the progeny virions to be passed on to adjacent uninfected cells. The evidence that cell-to-cell transmission occurs in influenza virus lead to the caution that local infection proceeds even when treated with neuraminidase inhibitors.

Introduction

It is generally accepted that viruses, released as *cell-free* virions from an infected cell, transmit to distant cells and tissues. This spreading pathway contributes to wide-ranged diffusion of *cell-free* viruses. However, in this spreading pathway, viruses are exposed to host anti-virus defense systems. In contrast, direct infection to a neighboring cell is considered to be beneficial for the virus in terms of evasion from the host anti-virus defense. There are two typical manners in infection to “*right next door*”: One is the virus transmission through cell-cell fusion by forming syncytium without production of progeny virions, and the other is mediated by virions without virus diffusion, generally designated cell-to-cell transmission [45,46].

The cell-cell fusion infection pathway is characteristic for a variety of virus such as paramyxoviruses, herpesviruses, some retroviruses, and so on. For example in the case of measles virus belonging to *Paramyxoviridae*, infection is initiated by the interaction of the viral hemagglutinin glycoprotein with host cell surface receptors. The virus penetrates into the cell through membrane fusion mediated by the interaction of the fusion glycoprotein. In later stages of infection, newly synthesized glycoproteins accumulate at the cell membrane resulting in fusion of the infected cell with neighboring cells by

producing syncytia. Thus, viruses can spread from cell to cell without producing *cell-free* virus particles.

The examples of the cell-to-cell transmission are diverse, and these mechanisms are dependent on pairs of viruses and host cells. Vaccinia virus particles bound on the filopodium of an infected cell are repelled toward neighboring uninfected cells by the formation of filopodia using actin filament [76]. The filopodia direct viruses to uninfected cells. Immunotropic viruses including retroviruses utilize an immunological synapse, designed as virological synapses for the cell-to-cell transmission [52-55]. Claudin-1 and occludin, components of tight junction, are involved in hepatitis C virus (HCV) entry through the cell-to-cell transmission [48,49]. The cell-to-cell transmission through tight junction is also observed in other viruses which infect epithelial layers [50,51]. These retroviruses and HCV remain on the surface of an infected cell even after budding. The uninfected cells adjacent to these infected cells can accept or take over viruses from the infected cell. Thus, the cell-to-cell transmission can be categorized into two manners based on the state of infecting viruses, either *cell-free* or cell-associated virions.

Influenza virus, belonging to the family of *Orthomyxoviridae*, is one of the most serious zoonotic pathogens and causes seasonal epidemics or periodic pandemics among

human beings around the world. The viral envelope consists of a lipid bilayer derived from cells that anchors three of viral transmembrane proteins, hemagglutinin (HA), neuraminidase (NA), and matrix protein 2 (M2). Influenza virus infection is initiated by the attachment of HA on virus particles to cell surface receptors containing sialic acids [4]. It has been known that the specific interaction between HA and sialic acid species is one of the determinants of the host range of influenza viruses [77]. Beside its role in the viral attachment, HA is also involved in intracellular fusion between viral envelope and host cell endosome membrane in the endocytotic pathway, by which the virus content is released inside the host cell [78]. The functional maturation of HA is mediated by the cleavage of HA into two disulfide-linked glycopolypeptides, HA1 and HA2 [79], accomplished by trypsin or trypsin-like proteases derived from host cells [80-83]. The membrane fusion is induced by a conformational change in the mature HA, which is triggered at low pH in the endosome, allowing viral ribonucleoprotein complexes to release into the cytoplasm [84,85]. Thus, HA plays a critical role in initiation and progression of influenza virus infection. Influenza virus NA possesses the enzymatic activity that cleaves α -ketosidic linkages between terminal sialic acids and adjacent sugar residues of cellular glycoconjugates [86]. The sialidase activity of NA removes terminal sialic acid residues from HA and NA proteins as well as host cell surface glycoproteins. Since the terminal

sialic acid of sialyloligosaccharides is critical for HA binding, the receptor-destroying activity of NA serves to counter the receptor-binding activity of HA. It is quite likely that this activity contributes to prevention of successive superinfection of an infected cell [87]. In the absence of the functional sialidase activity, progeny virions aggregate on the cell surface due to the HA receptor-binding activity and can not be released [19,88]. Thus, NA cleaves sialic acids from the cell surface and facilitates virus release from infected cells. However, it is not clear whether every progeny virion is released as *cell-free* virion to infect the uninfected cells after diffusion into the extracellular environment. Influenza viruses are generally transmitted as *cell-free* viruses from infected to uninfected cell but they may also infect through the cell-to-cell transmission, in particular during local lesion formation.

Here, we examined whether influenza virus transmits from an infected cell to adjacent uninfected cells without virus release. Live cell imaging techniques showed that a recombinant influenza virus, in which the *NA* gene was replaced with the *green fluorescence protein* gene, spreads from an infected cell to adjacent cells forming infected cell clusters. Furthermore, progeny virions remain associated on the surface of infected cell even after budding, and then progeny virions could be passed to adjacent uninfected cells.

Results

Influenza virus can spread in an NA-independent manner to adjacent cells.

To examine the transmission pathway of influenza virus, we performed immunofluorescence analyses by using anti-nucleoprotein (NP) polyclonal antibody. Influenza virus can form an infection center even in the presence of oseltamivir, a potent NA inhibitor (commercially known as Tamiflu) [89-91]. Oseltamivir at the concentration of 50 $\mu\text{g/ml}$ completely prevented the release of progeny influenza viruses (Figure 3-1A). Noted that a large number of single fluorescent foci caused by initial infection markedly expanded and formed cell clusters consisting of 5-10 infected cells in an MDCK cell monolayer (Figures 3-1B and 3-2), suggesting influenza virus can spread to some extent in the presence of oseltamivir. To verify that NA is not involved in this spreading, we generated an NA-deficient influenza virus by a reverse genetics method as described previously [92,93]. The NA-deficient influenza virus contains a mutated NA segment, in which the NA coding region including a sialidase catalytic domain was replaced with the *enhanced green fluorescent protein (EGFP)* gene [92]. By this replacement, the NA activity is eliminated from the recombinant influenza virus, and *EGFP* can be utilized as a marker for viral infections. Immunofluorescence analyses demonstrated that the NA-deficient influenza virus also forms infected cell clusters similarly to those formed by

wild-type influenza virus in the presence of oseltamivir (Figure 3-1B). The fluorescence pattern of NP overlapped with the localization of GFP derived from the *EGFP* gene of the NA-deficient influenza virus (Figure 3-3). Thus, NA-deficient influenza virus can be used to investigate the NA-independent infection pathway of influenza virus.

Next, we performed live cell imaging analyses to directly observe the infection time course of the NA-deficient influenza virus. The GFP fluorescence derived from the NA-deficient influenza virus first appeared in a single cell on an MDCK cell monolayer at 24 hours post infection. The virus started to spread from an infected cell to adjacent cells in 5-6 hours after the first appearance of a GFP-positive cell (Figure 3-4). The spreading rate was clearly faster than the rate of cell divisions. The mean doubling time of uninfected MDCK cells was 20-24 hours under the condition employed here, and it is expected that the proliferation speed would be much slowly because infected MDCK cells were maintained in the serum-free medium and formed cell monolayer at the high cell density. These suggest that NA-deficient influenza viruses may infect adjacent cells through the cell-to-cell transmission mechanism without apparent production of *cell-free* virions.

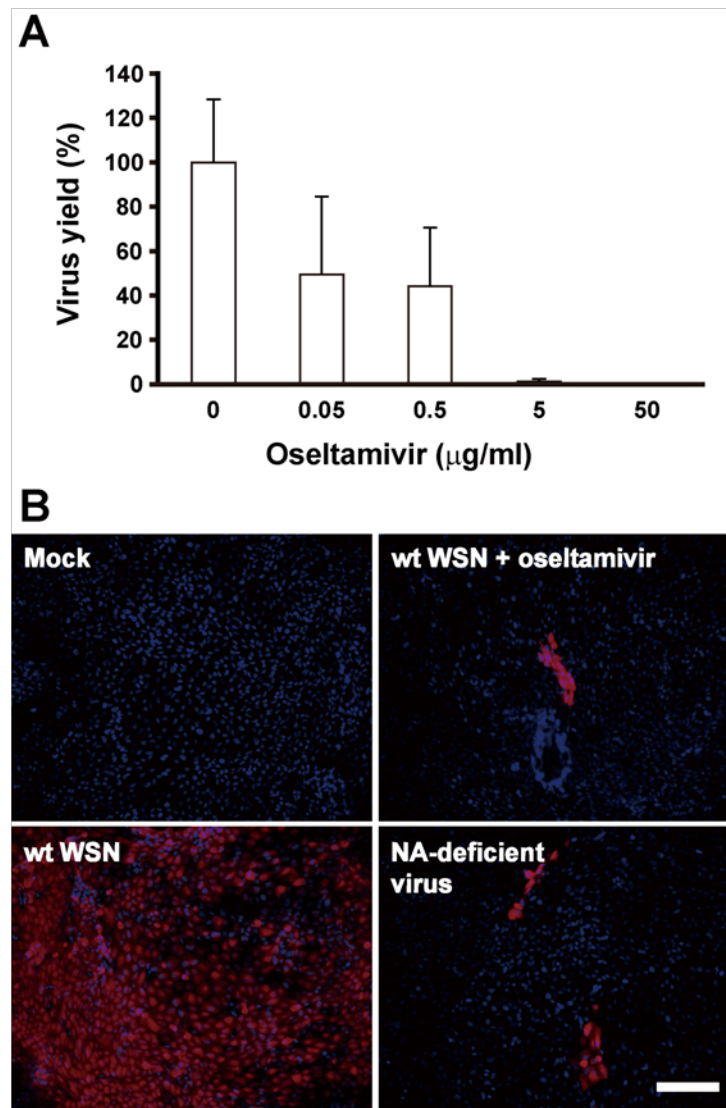


Figure 3-1. Influenza viruses can spread independent of the NA activity. (A) MDCK cells were infected with influenza virus A/WSN/33 at a multiplicity of infection (MOI) of 0.001 PFU per cell. At 48 hours post infection (hpi), culture supernatant was collected, and then its virus titer was determined by plaque assays. Each result was represented by a value relative to that in the absence of the drug. Error bars indicate standard deviation (s.d.) from 3 independent experiments. (B) Confluent MDCK cells were infected by wild-type influenza virus A/WSN/33 or NA-deficient influenza virus at MOI of 0.0001 in the presence or absence of 50 μg/ml oseltamivir phosphate. NA-deficient influenza virus was generated by reverse genetics as previously described [92]. After incubation at 37°C for 36 hours, immunofluorescence analyses were performed using anti-nucleoprotein (NP) polyclonal antibody and anti-rabbit IgG antibody conjugated to Alexa Fluor 568 (Invitrogen). Scale bar, 100 μm.

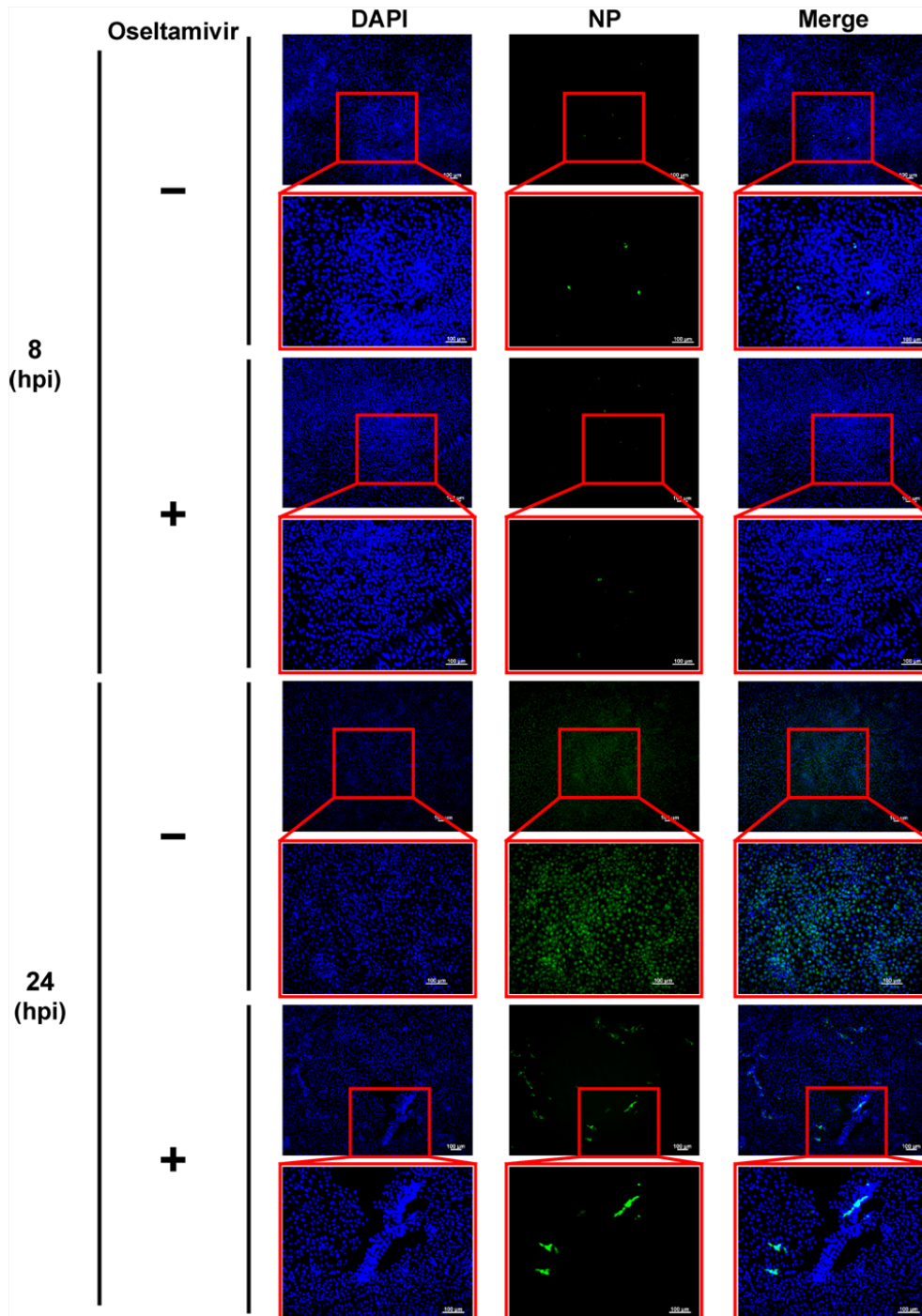


Figure 3-2. Formation of cell cluster caused by initial infection. MDCK cells were infected with influenza virus A/WSN/33 at moi of 0.0003 in the presence or absence of 50 μg/ml oseltamivir phosphate. After incubation for 8 and 24 h, immunofluorescence analyses were performed using anti-NP antibody and anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Invitrogen). Nuclear DAPI and viral NP staining patterns are shown in blue and green, respectively. Enlarged views are shown in red borders. Scale bar, 100 μm.

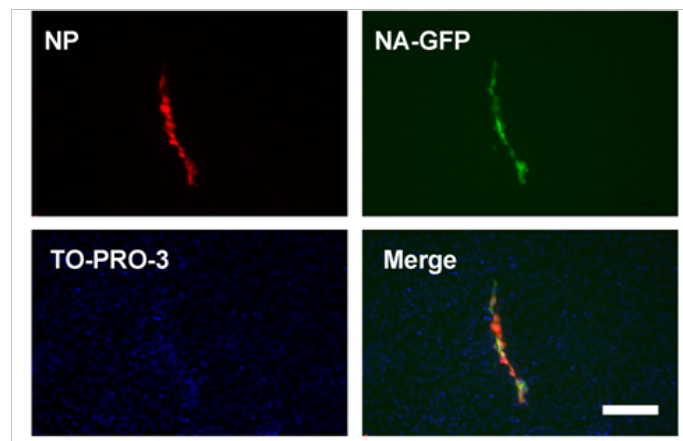


Figure 3-3. The expression of GFP derived from NA-deficient influenza virus overlapped with the localization of NP. MDCK cells were infected with NA-deficient influenza viruses at MOI of 0.0001. After incubation at 37°C for 48 hours, immunofluorescence analyses were performed using anti-NP antibody. Scale bar, 100 μ m.

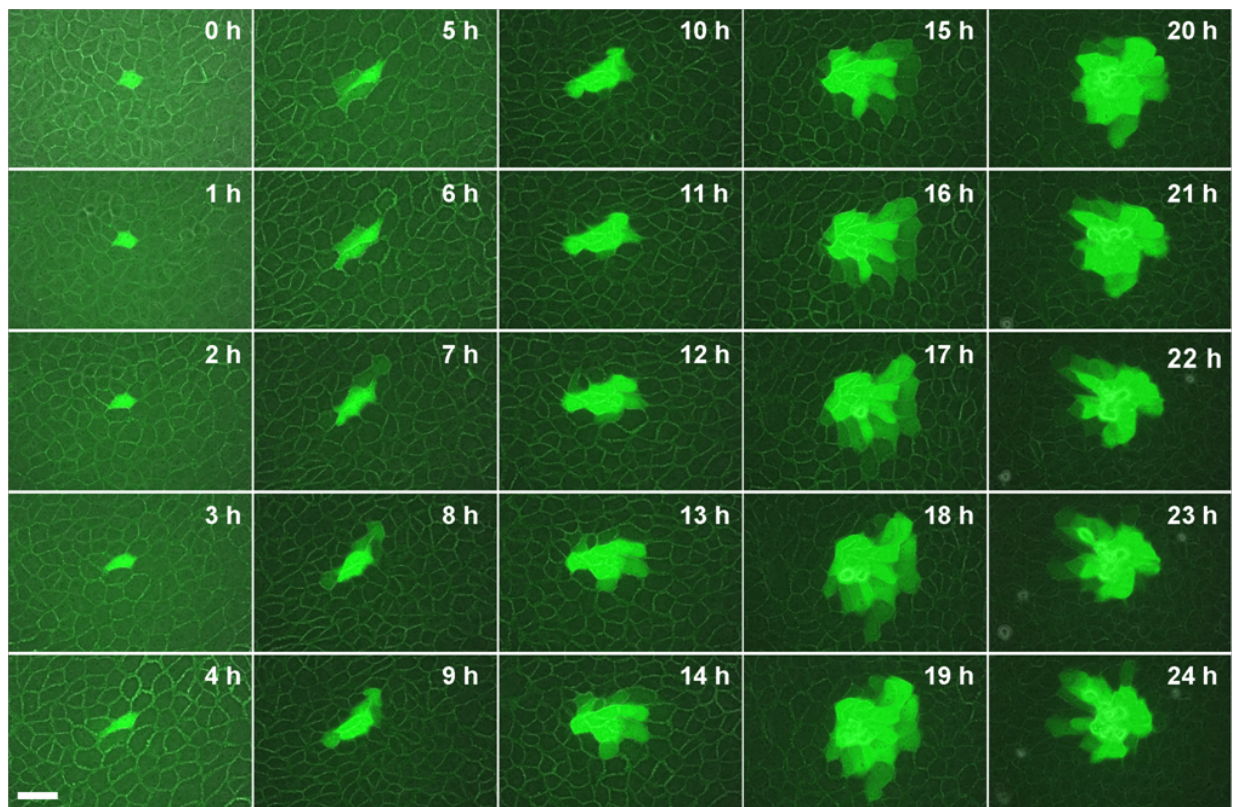


Figure 3-4. NA-deficient influenza virus spreads through cell-to-cell transmission. Confluent MDCK cells were infected with the NA-deficient influenza virus at MOI of 0.0001. After incubation at 37°C for 24 hours, a single GFP-positive cell, in which the recombinant virus replicated, was found at 1 hour after starting monitoring, and then this cell and its neighborhood were traced during the period from 24 hpi to 48 hpi at interval of 1 hour. Scale bar, 50 μ m.

Cell-to-cell transmission pathway of influenza viruses is less sensitive to neutralizing antibody

The cell-to-cell virus transmission pathway could be interpreted as one of viral evolving strategies to avoid neutralizing antibody responses [45,94,95]. Therefore, we examined the effect of neutralizing antibody on NA-deficient influenza virus. A polyclonal antibody with the neutralizing activity against influenza virus particles inhibited infection of *cell-free* viruses to less than 50% at the concentration of 0.03%, although the cell cluster formation was observed at the concentration less than 0.01%. On the other hand, the NA-independent transmission of the NA-deficient influenza virus was blocked only when neutralizing antibody was present at the concentration of 0.3% (Figure 3-5). These results indicated that the NA-independent transmission of influenza viruses is less sensitive to the neutralizing antibody.

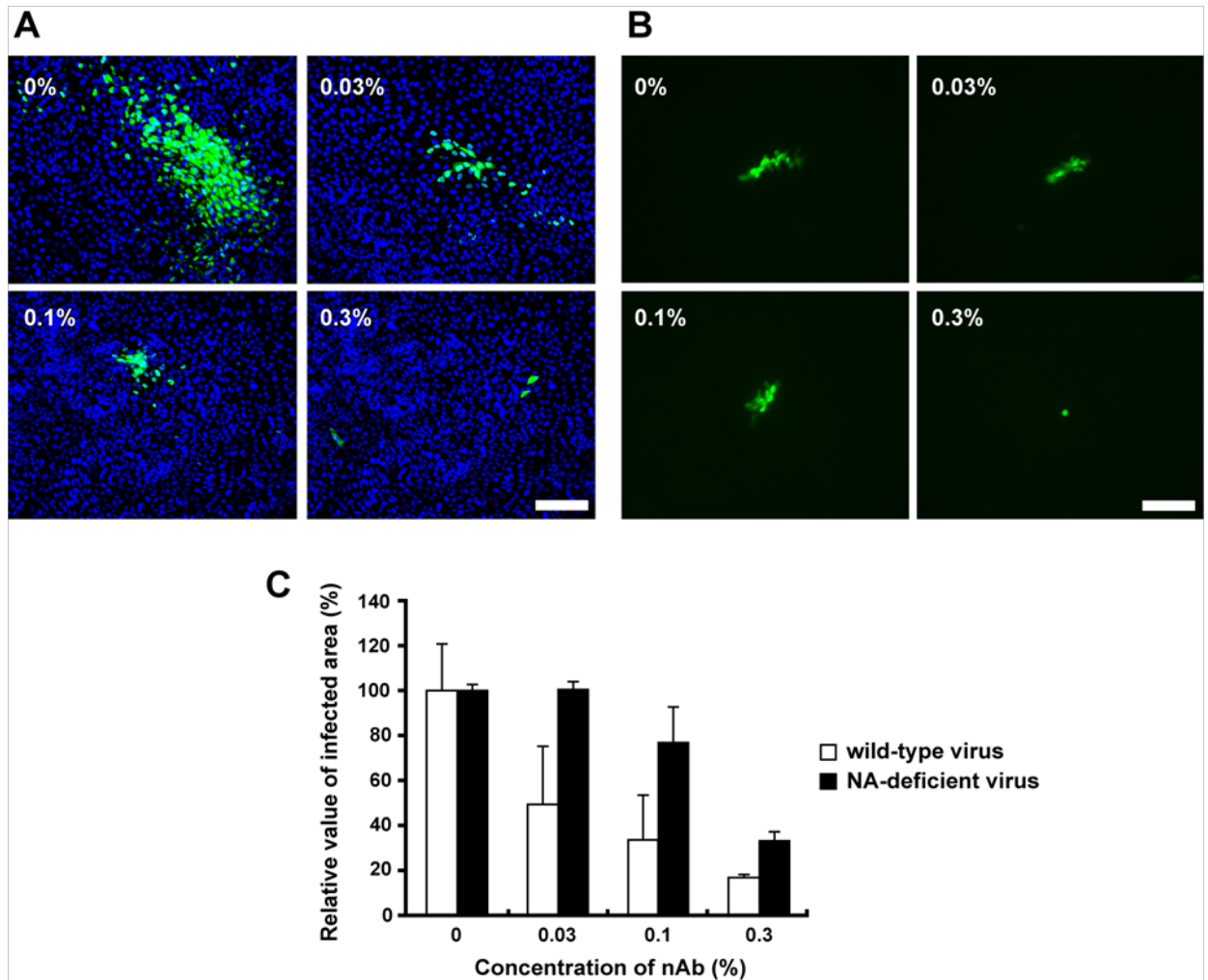


Figure 3-5. The cell-to-cell transmission of the NA-deficient influenza virus is less sensitive to the neutralizing antibody. (A) Infection of the wild-type and (B) NA-deficient influenza virus were performed in the presence or absence of antiserum containing neutralizing antibodies. Immunofluorescence analyses were performed with cells infected with wild-type influenza virus at 18 hpi using anti-NP antibody and anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Invitrogen). GFP fluorescence derived from the recombinant virus was observed at 36 hpi. Scale bar, 100 μ m. (C) The level of viral spreading was indicated in the graph by measuring NP and GFP derived from wild-type and NA-deficient virus, respectively. Five different microscope fields were taken randomly, and then the intensity of green color was analyzed with ImageJ NIH image processing software. Each result was represented by a value relative to that in the absence of neutralizing antibodies. Error bars indicate s.d. from 3 independent experiments.

NA-independent transmission of influenza virus is HA-dependent.

Next, to investigate the mechanism of NA-independent transmission of influenza virus, we examined whether HA is involved in this transmission. In the absence of the NA activity, virus spreading from an infected cell to adjacent cells was dramatically suppressed by omission of trypsin, essential for maturation of HA, from the experimental condition (Figure 3-6A). The GFP fluorescence derived from NA-deficient influenza virus appeared in a single cell at 24 hours post infection. However, this virus did not spread, but rather disappeared during subsequent 24 hours (Figure 3-7). These observations indicate that the NA-independent cell-to-cell transmission of influenza virus is dependent on HA maturation mediated by trypsin, as is the case for the general *cell-free* transmission of this virus.

To clarify whether virus particles or viral RNP complexes are transmitted to adjacent cells, we examined the effect of amantadine on the cell-to-cell transmission of influenza virus. Amantadine inhibits the early step of uncoating of influenza virus RNP from virion in endosomes [96,97]. For this study, other influenza virus strain, influenza virus A/Udorn/72, was used instead of influenza virus A/WSN/33 because influenza virus A/WSN/33 is highly resistant to amantadine [98]. We confirmed that influenza virus A/Udorn/72 is sensitive to oseltamivir (Figure 3-8) and could also spread via cell-to-cell

transmission independent of the NA activity as did for influenza virus A/WSN/33 (Figures 3-1B and 3-6B). In the case of a single administration of amantadine, fluorescent foci derived from infected cells scattered, and the number of single foci was greatly decreased compared with that in the absence of the drugs. In contrast, a single administration of oseltamivir, fluorescent foci formed some clusters and expanded in a time-dependent manner (Figure 3-6B). This dissimilarity of inhibitory manner was caused by the difference of the sites of action between amantadine and oseltamivir. Amantadine inhibits the replication of influenza A virus by preventing the translocation of vRNP complexes from endosomes to the cytoplasm, whereas oseltamivir has no effects on viral replication itself but inhibits the release of *cell-free* virions from infected host cells. We investigated the inhibitory effect of amantadine on the cell-to-cell transmission of influenza viruses. The formation of infected cell clusters was observed with co-administration of amantadine and oseltamivir, as well as with a single administration of oseltamivir (Figure 3-6B). However, the quantitative analysis revealed that the size of infected cell clusters with the co-administration were decreased as compared to that with oseltamivir alone (Figure 3-6C). These observations indicated that the NA activity-independent cell-to-cell transmission of influenza virus was susceptible to the inhibitory effect of amantadine, suggesting that the cell-to-cell transmission undergoes

through endocytosis but vRNP complex itself is not incorporated in the infected cells by adjacent cells.

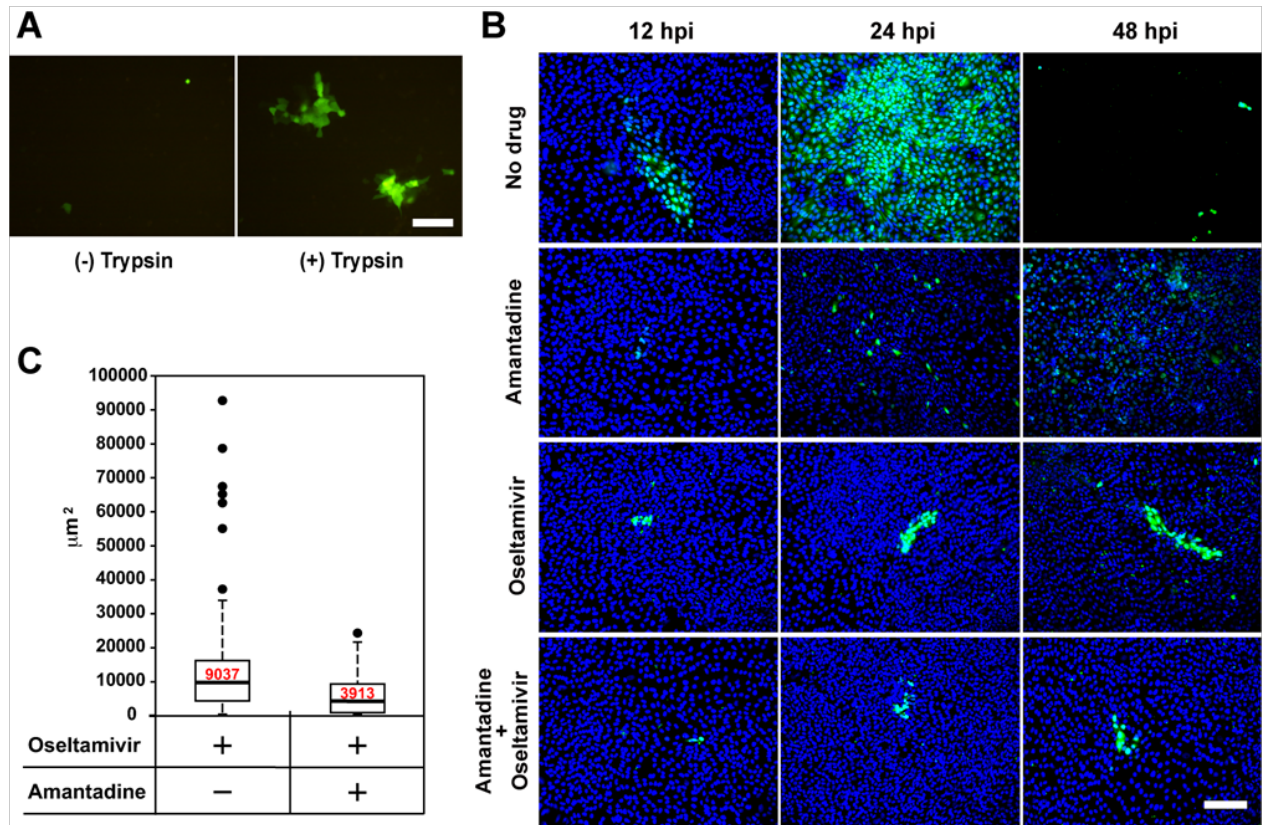


Figure 3-6. The cell-to-cell transmission of the NA-deficient influenza virus requires functional HA. (A) Confluent MDCK cells were infected with the NA-deficient influenza virus at MOI of 0.0001 in the presence or absence of 1 μg/ml trypsin. GFP fluorescence derived from the recombinant virus was observed at 36 hpi. Scale bar, 100 μm. (B) MDCK cells were infected with influenza virus A/Udorn/72 at moi of 0.0001 in the presence or absence of 50 μM amantadine or 50 μg/ml oseltamivir phosphate. Amantadine at the concentration of 50 μM almost completely inhibited the production of progeny virions (data not shown). After incubation for 12, 24, and 48 h, immunofluorescence analyses were performed using anti-NP antibody and anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Invitrogen). Viral NP and nuclear DAPI staining are shown in green and blue, respectively. Scale bar, 100 μm. (C) Median sizes of clusters were shown as box plots summarizing sizes of 60 individual infectious foci formed in the presence of oseltamivir alone, or both oseltamivir and amantadine. Immunofluorescence analyses were performed as described in (B) at 24 hpi. Boxes enclose the lower and upper quartiles; thick horizontal lines represent the median; dashed lines indicate the extreme values; and black dots are outliers of individual infectious foci. The size of infectious foci was measured with AxioVision Release 4.7.2 imaging software (Carl Zeiss). Median sizes shown in red letters were clearly different from each other ($p < 0.01$).

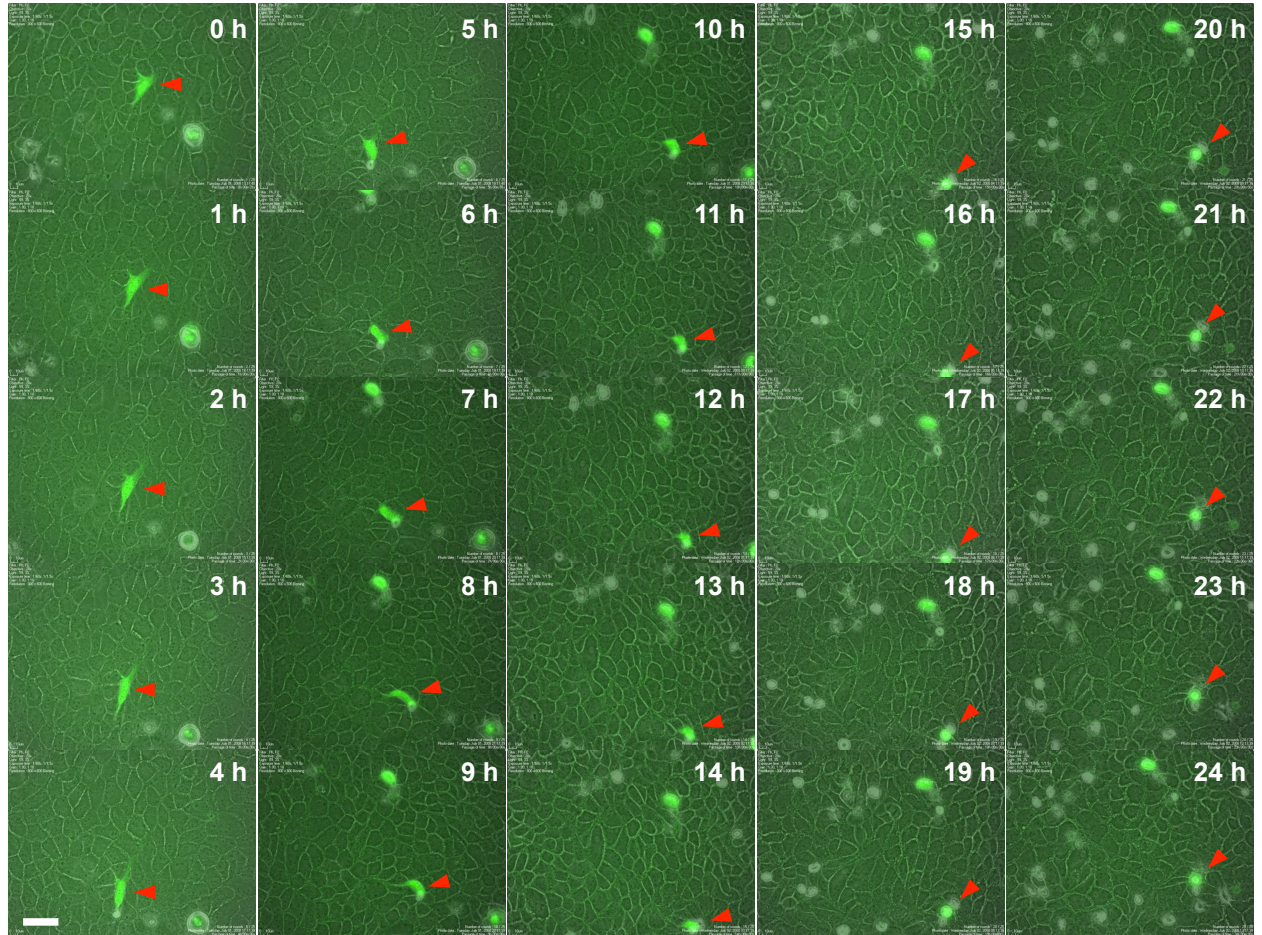


Figure 3-7. NA-deficient influenza virus does not spread in the absence of trypsin. Confluent MDCK cells were infected with the NA-deficient influenza virus at MOI of 0.0001 in the absence of trypsin. After incubation at 37°C for 24 hours, a single GFP-positive cell was detected, and then this cell and neighborhood cells was traced during the period from 24 hpi to 48 hpi at interval of 1 hour. Live cell imaging data analyses were performed by Biostation ID (GE healthcare). An infected cell is represented by red arrowhead. Scale bar, 50 μ m.

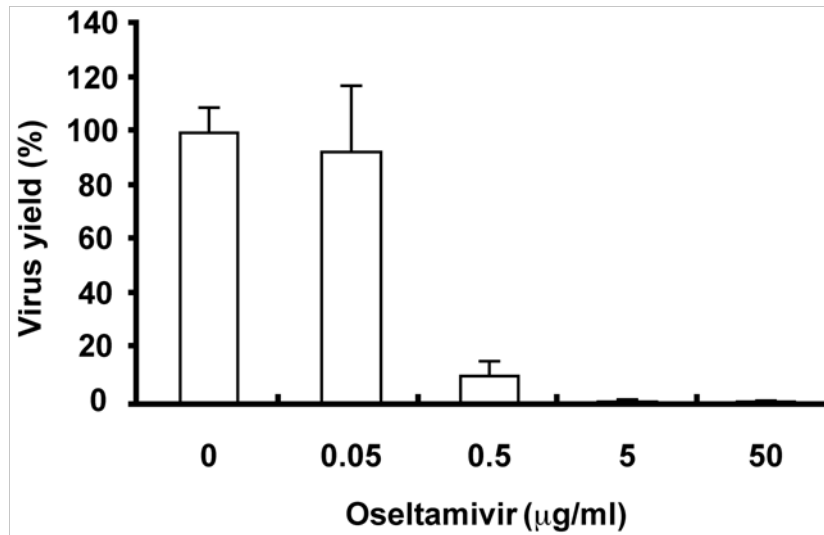


Figure 3-8. Influenza virus A/Udorn/72 was sensitive to oseltamivir. MDCK cells were infected with influenza virus A/Udorn/72 at a MOI of 0.001 PFU per cell. At 36 hpi, the culture supernatant was collected, and then its virus titer was determined by plaque assays. Each result was represented by a value relative to that in the absence of the drug. Error bars indicate s.d. from 3 independent experiments.

Cell-to-cell transmission occurs on the apical cell membrane.

The virus transmission undergoes from infected to uninfected cells through either basolateral [99-101] or apical [102-105] sides. In the case of influenza virus, *cell-free* progeny virions are released only from the apical surface of polarized epithelial cells [57]. This releasing polarity is achieved by directed transport of viral membrane proteins to the apical plasma membrane [106]. Indeed, that HA and NA glycoproteins are associated with lipid rafts, and the raft association has been implicated in apical transport [107,108].

To determine whether or not the cell-to-cell transmission of the NA-deficient influenza virus occurs on the apical surface, we performed transwell assays in the presence of the neutralizing antibody to influenza A viruses. The neutralizing antibody was added to infected MDCK cell monolayer from apical or basolateral side, and the inhibitory effect on the spread of GFP fluorescence derived from the recombinant virus was examined. Addition of high concentrations of the neutralizing antibody from the apical side blocked the cell-to-cell transmission of the NA-deficient influenza virus, whereas the addition from the basolateral side had no effect (Figure 3-9). These observations indicated that the polarity in the influenza virus budding in the cell-to-cell transmission pathway is apical.

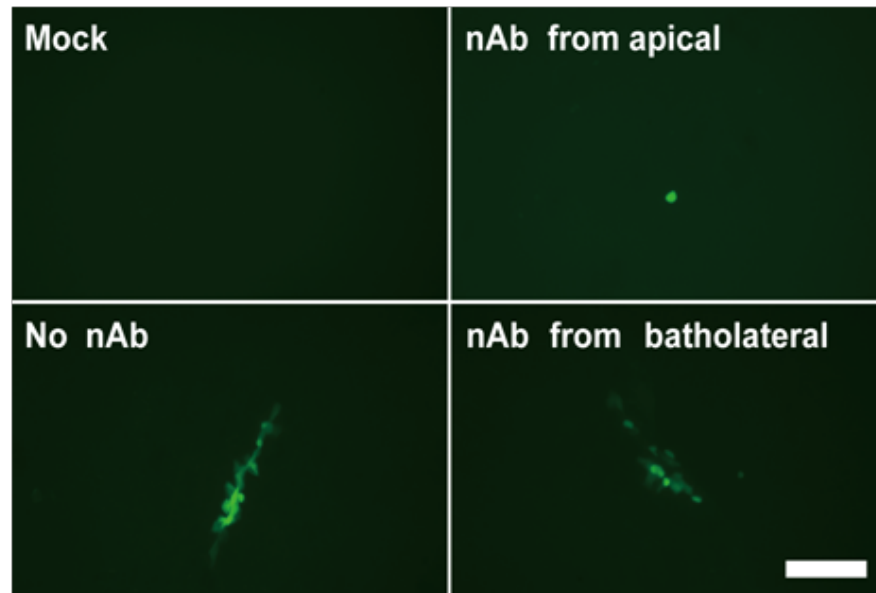


Figure 3-9. The cell-to-cell transmission of the NA-deficient influenza virus occurs the apical cell surface. Confluent MDCK cells were prepared in transwell inserts and infected with the NA-deficient influenza virus at MOI of 0.0001 in the presence or absence of 0.3% (v/v) antiserum containing neutralizing antibodies (nAb) to influenza A virus. After virus adsorption, the antiserum was added from apical or basolateral side. GFP fluorescence derived from the recombinant virus was observed at 36 hpi. The antiserum added from the apical side could markedly block the cell-to-cell transmission of the NA-deficient influenza virus, whereas the antiserum added from the basolateral side could not. Scale bar, 100 μ m.

Influenza viruses can not re-infect previously infected cells

Previous report showed that influenza viruses were refractory to superinfection with a second cell-free virus [87]. In the case of the cell-to-cell transmission of influenza virus in the presence of oseltamivir, it is possible that a progeny virion is temporarily bridged by HA between an infected cell and adjacent uninfected cells, since viruses cannot be released from infected cell surface due to the inhibition of the NA activity by oseltamivir. The cell-associated progeny virion may have an opportunity to re-infect the previously infected cell, compared to a cell-free progeny virion in the general spreading. Thus, we examined whether influenza viruses can infect the cell which had already been infected, using *ts53* mutant and wild-type influenza virus A/WSN/33. *ts53* virus has a substitution mutation from U to C at the nucleotide position of 701 in the PA gene. This substitution introduces an amino acid change from wild-type Leu 226 to Pro 226 and gives a defect in the viral genome replication process [109,110]. At first, cells were infected with *ts53* virus at moi of 10, and after incubation for 0, 2, 4, 6, and 8 hours, cells were superinfected with wild-type virus at moi of 10. The amount of segment 3 viral RNA (vRNA) encoding PA was determined quantitatively by RT-PCR. Then, using a mutated primer for PCR, we could introduce a *Stu* I site only in the PCR products derived from the wild-type sequence

(Figure 3-10A). Thus, DNA fragments amplified from the wild-type and *ts53* could be distinguished by *Stu* I digestion. The digested DNA fragments containing 220 and 199 base pairs derived from *ts53* and wild-type, respectively, were separated through PAGE. After 6 hours or later post infection, re-infection with the second challenging virus hardly occurs in the absence of oseltamivir. However, in the presence of oseltamivir, appearance of wild-type fragment suggests that the re-infection had occurred (Figure 3-10B). The result indicates that progeny virus particles remain on the surface of infected cell even after budding, and can infect the cell previously infected, as well as uninfected cells adjacent to the infected cell, when oseltamivir is present.

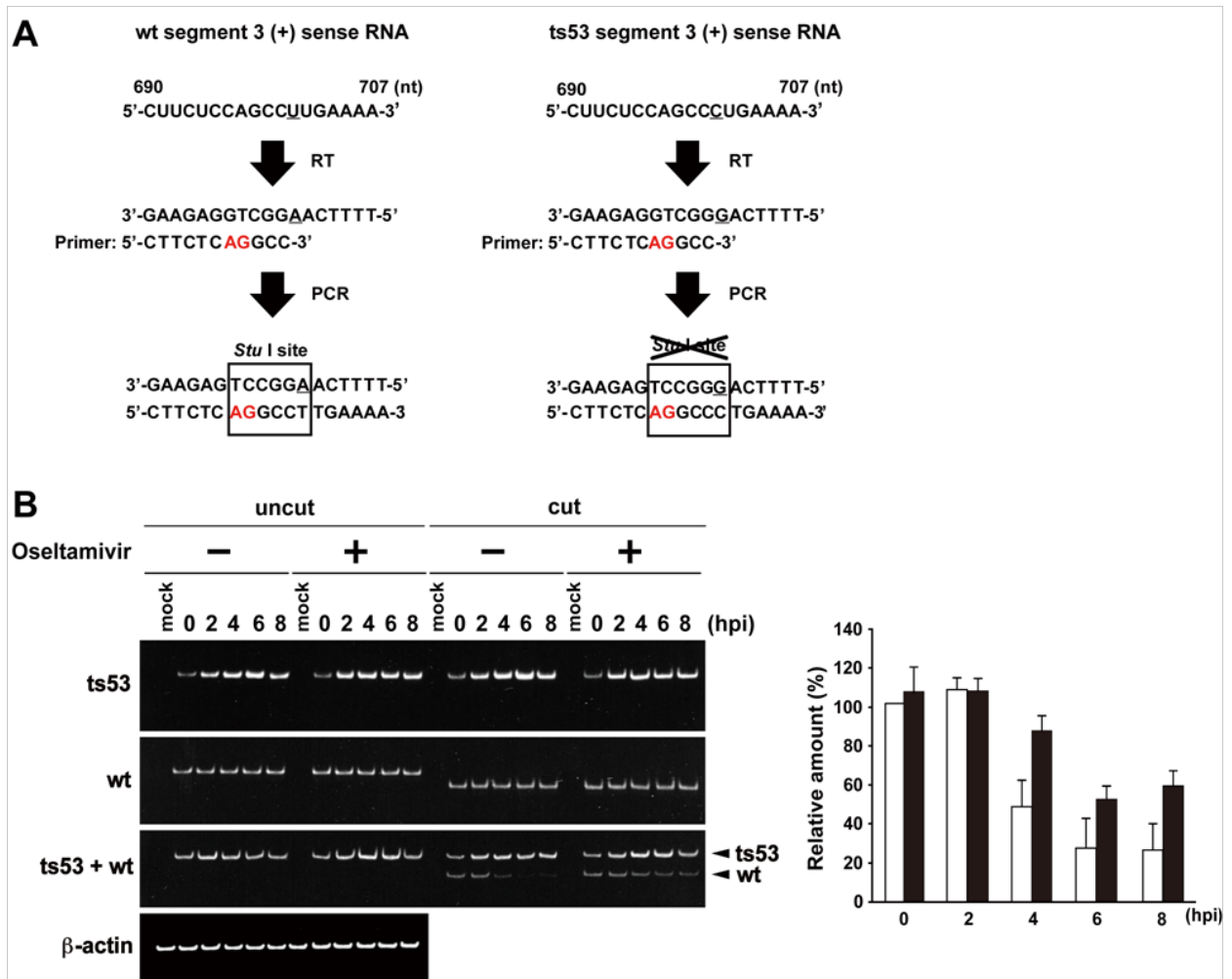


Figure 3-10. Influenza viruses can not re-infect previously infected cells. (A) A method for determination of the amount of segment 3 genome derived from *ts53* and wild-type. Total RNA was reverse-transcribed with the primer PA-895-rev, which is complementary to the segment 3 positive-sense RNA. The cDNA was amplified by PCR using primers, PA-895-rev and PA-695-cut partially corresponding to segment 3 positive sense RNA between the nucleotide sequence positions 678 to 700 except for 696 and 697, which are shown in red letters. Since segment 3 of *ts53* has a substitution mutation from U to C at the nucleotide position of 701, the PCR product derived from wild-type could be digested by *Stu* I but not that from *ts53*. Then, PCR products were digested with *Stu* I and separated through 8% PAGE. (B) Detection of the genome of the segment 3 derived from *ts53* or wild-type. At 3 hours post superinfection of wild-type virus, total RNA was extracted, and semi-quantitative RT-PCR was performed. Subsequently, the amplified DNA products were digested with *Stu* I and separated through 8% PAGE. Large and small fragments derived from *ts53* and wild-type viruses were 220 and 199 base pairs, respectively. The relative amount of wild-type segment 3 to that at 0 hour in the absence of oseltamivir phosphate was shown in the graph. Error bars indicate S.D. from 3 independent experiments. White bar, in the absence of oseltamivir phosphate; black bar, in the presence of oseltamivir phosphate.

Discussion

With the exception for the virus which spreads through the cell-cell fusion transmission, virus infection is initiated by the binding of *cell-free* virions to their host cells. Recently, the virus transmission mechanism from an infected cell to adjacent cells without virus diffusion into the extracellular environment is highlighted from the aspect of its significance in virus spreading in the presence of antibodies [45,46]. This antibody-insensitive pathway is often called cell-to-cell transmission [45]. The cell-to-cell transmission may be categorized into two pathways, *i.e.*, transmission of *cell-free* virions to adjacent uninfected cells, and transmission of progeny virions associated on the surface of an infected cell even after budding through narrow synaptic space between an infected cell and adjacent uninfected cells. As an example of the former mechanism, *cell-free* vaccinia virus particles associated with the filopodium of an infected cell are repelled toward neighboring uninfected cells by inducing the formation of actin filament [76]. Several cases have been reported for the latter mechanism: Immunotropic viruses including retroviruses utilize the immunological synapses [52-55]. Immune cells are not constitutively polarized, but contain the machinery that directs their secretory apparatus towards a cell that is involved in an immunological synapse. This machinery can be subverted by retroviruses containing human immunodeficiency virus

(HIV). An HIV-infected cell can polarize viral budding towards a target cell expressing receptor through a structure called a virological synapse. Virions bud from an infected cell into a synaptic cleft, from which they fuse with the target-cell plasma membrane [111-114]. The progeny virions of HCV are trapped between infected and uninfected cell membranes at the tight junction. Using Claudin-1 known as a component of the tight junction and one of the entry factors of HCV [48], virions fuse with and penetrate uninfected target cells [94]. Therefore, HCV may acquire the ability to spread within polarized liver epithelium. Thus, the cell-to-cell transmission certainly plays significant roles for the dissemination of several enveloped viruses. However, the cell-to-cell transmission of influenza virus has not been discussed well. Here, we have shown that influenza virus spreads by forming infected cell clusters even in the presence of an NA inhibitor. Live cell imaging clearly showed that influenza virus lacking the NA activity spreads from an infected cell to adjacent cells through the cell-to-cell transmission mechanism (Figure 3-4). This was also the case for wild-type influenza virus during early phases of infection (Figure 3-6B). In the cell-to-cell transmission of influenza virus, progeny virions could remain associated with the surface of infected cell even after budding, and then these progeny virions can be passed on to adjacent uninfected cells.

We showed that the cell-to-cell transmission of the NA-deficient influenza virus

depends on functional HA. The viral spreading was dramatically suppressed without HA activation by trypsin treatment (Figure 3-6A). Moreover, the cell-to-cell transmission was also blocked by amantadine, which inhibits the acidification of endosomes required for uncoating of influenza virus particles in endosomes [96,97]. These findings indicate that functional HA and endosome acidification by M2 ion channel are required for the cell-to-cell influenza virus transmission, thereby allowing viruses to enter the adjacent cells through the endocytotic pathway (Figure 3-6).

Our findings showed that the NA-deficient influenza virus is not diffused into the extracellular environment. The viral spreading in the absence of oseltamivir appears to be much faster compared to the viral spreading in the presence of the drug, suggesting that NA could be involved in determination of spreading speed (Figure 3-6B). The NA activity prevented progeny virions from entering cells which virus came from (Figure 3-10), implying that progeny virus particles should be transmitted to adjacent uninfected cells. The cell-to-cell transmission started in early phase of infection, and the virus spread through diffusion of *cell-free* viruses (Figure 3-6B). Indeed, it was reported that the cell-to-cell transmission is a rapid spreading pathway in the case of vaccinia virus [76]. Vaccinia virus induces a blocking mechanism of superinfection and thereby infects to adjacent uninfected cells efficiently. In early phases of vaccinia virus infection, viral

proteins A33 and A36 are expressed at the infected cell surface. Once *cell-free* virus particles contact the filopodium, the A33/A36 complex induces the formation of actin filament, which causes this superinfected virion to be repelled toward uninfected cells [76]. Influenza viruses can re-infect the cells previously infected in the presence of oseltamivir (Figure 3-10), suggesting that a progeny virion may be bridged by HA between infected and adjacent uninfected cells temporarily (Figure 3-11). Thus, in the case of the cell-to-cell transmission of influenza virus, we propose that progeny virions associated with the surface of infected cells even after budding are directed to adjacent uninfected cells.

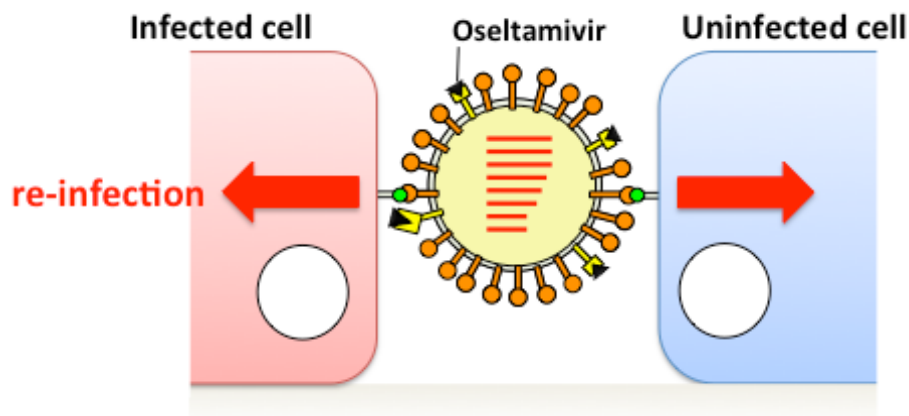


Figure 3-11. Working hypothesis for cell-to-cell transmission of influenza virus.

In cell-to-cell transmission, influenza virus may be bridged by HA between infected and adjacent uninfected cells temporarily. The progeny virion can re-infect the cells previously infected in the presence of oseltamivir.

The cell-to-cell transmission mechanism of influenza virus is distinctly different from that of vaccinia virus in the infecting virus status: Infected cell-associated

virions and *cell-free* virions are involved in the cell-to-cell transmission of influenza virus and vaccinia virus, respectively. The strategy for influenza virus appears to be similar to that for HCV. HCV progeny virions budded from an infected cell are trapped between infected and uninfected adjacent cell membranes at the tight junction. HCV virions then, enter into adjacent cells through endocytosis and low pH-dependent membrane fusion using Claudin-1 [48]. The cell-to-cell transmission of influenza virus also required functional HA and endosome acidification by M2 ion channel. However, it has not been reported that HCV has a gene encoding a receptor destroying enzyme similar to NA of influenza virus. We speculated that HCV progeny particles are bridged between infected and adjacent uninfected cells temporarily like influenza virus in the presence of oseltamivir. Progeny influenza virus particles could be transmitted to adjacent uninfected cells efficiently in the presence of the NA activity, suggesting that the cell-to-cell transmission of influenza virus is more strategic than that of HCV.

Our findings raise an interesting question as to what is the biological significance of cell-to-cell transmission for influenza virus infection *in vivo*. Until now, it had been believed that influenza virus was released from infected cells as *cell-free* virions and then spread from cell to cell as well as from organism to organism. The transmission mode by *cell-free* virions undergoes the extremely high-speed of its diffusion and causes epidemic

or pandemic infection. The tropism in an infected animal body is generally restricted to respiratory tract or lung and its periphery, and the requirement of a trypsin-like protease has been generally described for the reason of the restriction. It is possible that the cell-to-cell transmission mode may play a significant role for the virus spreading inside of organism, although *cell-free* influenza virions are causative of high-speed spreading. At the least, the limited but distinct level of infection followed by replication could provide some opportunity to generate influenza virus variants. It is an open question whether the cell-to-cell transmission mode is involved in the pathogenesis caused by influenza virus infection *in vivo*.

The existence of cell-to-cell transmission pathway gives a caution when NA inhibitors are used, because NA inhibitors may not be sufficient to completely block the spread of influenza virus in local microenvironments. Since this cell-to-cell transmission pathway exists, development of antiviral therapeutic strategies in addition to NA inhibitors is highly recommended.

Materials and Methods

Cells and viruses

Madin-Darby canine kidney (MDCK) cells were kindly gifted by A. Ishihama (Hosei University), and maintained in minimal essential medium (MEM) (Nissui) containing 10% fetal bovine serum. Human embryonic kidney 293T cells were kindly gifted by Y. Kawaoka (University of Tokyo), and maintained in Dulbecco modified Eagle medium (DMEM) (Nissui) supplemented with 10% fetal bovine serum. Influenza virus A/Udorn/72 was grown in allantoic sacs of 11 day-old embryonated eggs (MIYAKE HATCHERY). Wild-type influenza virus A/WSN/33 and *ts53* mutant were used after single-plaque isolation. MDCK cells were infected with influenza virus A/WSN/33 or *ts53* at a multiplicity of infection (MOI) of 0.1 PFU/cell, and incubated at 37°C and 34°C, respectively. After incubation for 24 h, the culture fluid was harvested and centrifuged at $1,700 \times g$ for 10 min. The virus suspension was stored at -80°C until use.

Antibodies

The production of rabbit polyclonal anti-NP antibody was described previously [115], and this antibody was used as a primary antibody for indirect immunofluorescence

assay. A goat anti-rabbit IgG antibody conjugated to Alexa Fluor 488 or Alexa Fluor 568 was purchased from Invitrogen and used as a secondary antibody for indirect immunofluorescence assay. A polyclonal antibody against influenza A virus was obtained from 2-month-old female rabbit immunized with 250 µg of purified virions of influenza virus strain A/Puerto Rico/8/34 [116]. The generation of antibodies was boosted three times and used as neutralizing antibodies to block the influenza virus infection.

Determination of the inhibition effect of oseltamivir on virus production

MDCK cells were infected with influenza virus A/WSN/33 at a multiplicity of infection (MOI) of 0.001 PFU per cell. After virus adsorption at 37°C for 1 hour, the cells were washed with serum-free MEM and incubated at 37°C with maintenance medium (MEM containing vitamins and 0.1% BSA) containing oseltamivir. At 48 hours post infection (hpi), culture supernatant was collected, and then its viral titer was determined by plaque assays.

Generation of neuraminidase (NA)-deficient viruses

An NA-deficient influenza virus possessing the terminal sequences of NA

segment but lacking the NA coding region, which was replaced with *enhanced green fluorescent protein (EGFP)* gene, was generated by reverse genetics as described previously [92,93]. For reverse genetics, we used plasmids containing cDNAs of the influenza virus A/WSN/33 viral genome under the control of the human RNA polymerase I promoter (referred to as Pol I plasmids). Briefly, 293T cells were transfected with seven Pol I plasmids for production of all vRNA segments of influenza virus A/WSN/33 and one for the mutant NA vRNA segment containing *EGFP* ORF, together with protein expression vectors for PB2, PB1, PA, and NP controlled by the chicken β -actin promoter (pCAGGS). TransIT-293 (Mirus) was used for transfection. At 24 hours post transfection, recombinant viruses were harvested from the cell surface using bacterial NA derived from *Clostridium perfringens* (sigma). MDCK cells were infected with harvested recombinant viruses treated with *N*-tosyl-L-phenyl-alanine chloromethyl ketone (TPCK)-trypsin (1 μ g/ml). After confirmation of GFP fluorescence derived from amplified recombinant virus genomes at 48 hours after infection, the recombinant viruses on the cell surface were collected using bacterial NA (Figure 3-12). The viral titer of recombinant viruses was determined by counting the number of infected foci using a fluorescence microscopy (Carl Zeiss).

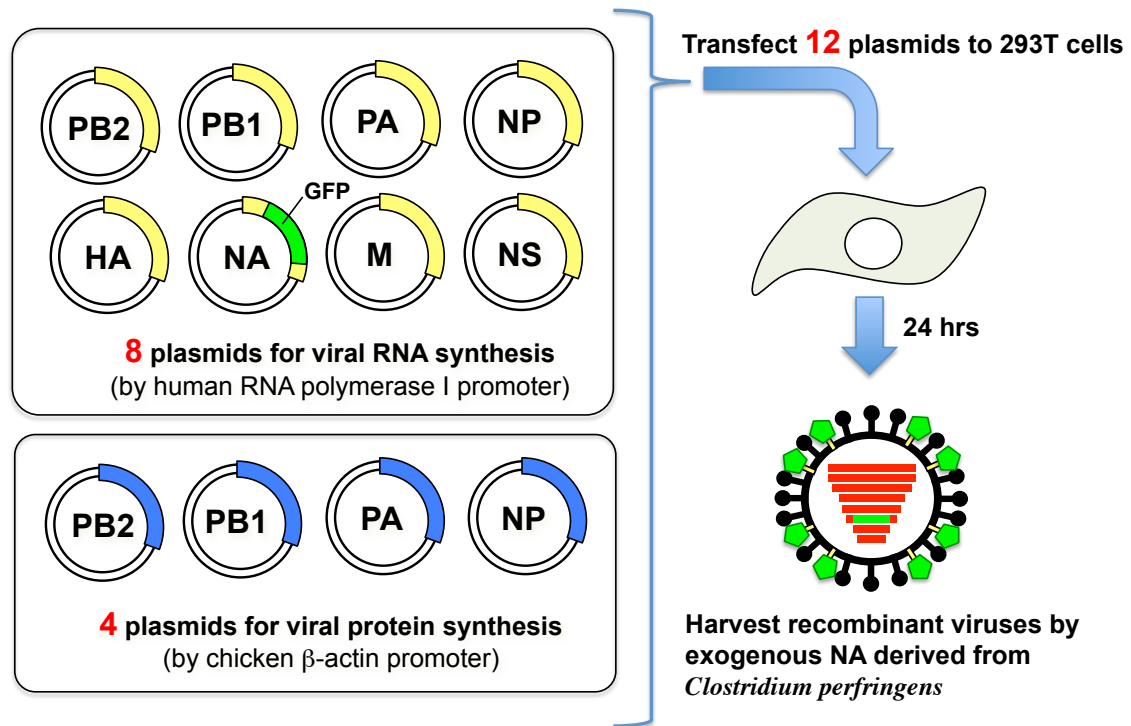


Figure 3-12. Experimental scheme of reverse genetics to generate NA-deficient viruses.

Indirect immunofluorescence assay

Cells on coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.2% NP-40 in PBS. The coverslips were soaked in 1% bovine serum albumin in PBS, and then incubated at room temperature for 1 hour with a primary antibody. After being washed twice with PBS, the coverslips were incubated at room temperature for 1 hour with a secondary antibody. The coverslips were then incubated at room temperature for 5 min with 3 μ M 4',6'-diamidino-2-phenylindole

(DAPI) and finally mounted on glass plates, and cells were observed under the fluorescence microscope.

Live cell imaging analyses

Living cells were analyzed using BioStation ID system (GE Healthcare). Confluent MDCK cells were infected with the NA-deficient influenza virus at the multiplicity of infection (MOI) of 0.0001 in the presence or absence of 1 $\mu\text{g/ml}$ TPCK-trypsin. At 24 hours post infection, culture dishes containing infected cells were set into the chamber of BioStation ID system, which was maintained at 37°C under 5% CO₂ and 95% humidity. Then, images were acquired during next 24 hours at interval with 1 hour. The excitation wavelength was controlled by a manual filter wheel equipped with filters suitable for enhanced green fluorescence protein (EGFP).

Transwell assay

Confluent MDCK cell monolayer was prepared on transwell inserts (BD Falcon, pore size 0.4 μm) and infected with the NA-deficient influenza virus at MOI of 0.0001. After virus adsorption at 37°C for 1 hour, the cell monolayer was washed with serum-free MEM, and maintenance medium was added into both sides within the transwells. The

neutralizing antibody to influenza A virus was added into the inside or the outside of transwell inserts with the maintenance medium. Subsequently, cells were incubated at 37°C for 36 hours followed by analyses using the fluorescence microscopy.

RT-PCR

ts53 virus has a substitution mutation from U to C at the nucleotide position of 701 in the *PA* gene. This substitution introduces an amino acid change from wild-type Leu 226 to Pro 226 and gives a defect in the viral genome replication process [110]. However, under the permissive temperature, the level of viral genome replication is no difference between wild-type and *ts53* [109]. To discriminate the genome of wild-type and that of *ts53*, total RNA was reverse-transcribed by reverse transcriptase (TOYOBO) with PA-895-rev (5'-TTAATTTTAAGGCATCCATCAGCAGG-3'), which is complementary to the segment 3 positive sense RNA. The cDNA was amplified by PCR using primers, PA-895-rev and PA-695-cut (5'-TCTCCCGCCAAACTTCTCAGGCC-3') partially corresponding to segment 3 positive sense RNA between nucleotide sequence positions 678 to 700 except for nucleotide positions 696 and 697. Since segment 3 of *ts53* has a substitution mutation from U to C at the nucleotide position of 701, the PCR product derived from wild-type was digested by *Stu* I but not that from *ts53*. After PCR

reactions, PCR products were digested with *Stu* I and separated through PAGE. Large and small fragments derived from *ts53* and wild-type viruses were 220 and 199 base pairs, respectively. DNA was stained with GelRed (BIOTIUM) and visualized by UV illumination.

Chapter 4

SUMMARY OF FINDINGS, DISCUSSION AND FUTURE DIRECTIONS

Summary of findings and discussion

The examples of cell-to-cell transmission pathway, which is mediated by virions without virus diffusion, were reported in HCV, immunotropic viruses including retroviruses, and some neurotropic viruses. However, the cell-to-cell transmission of influenza virus has not been discussed well. This study has shown that influenza viruses lacking the neuraminidase activity were capable of spreading from an infected cell to adjacent uninfected cells via the cell-to-cell transmission mechanism. Live imaging technique clearly demonstrated the cell-to-cell transmission of influenza viruses in a neuraminidase independent manner.

In the case of cell-to-cell transmission of HCV, virus particle can be transported via tight junction and it has been explained as one of the viral strategy to escape from the host immune defence. In contrast, influenza virus particles were transported via apical surface and its transmission were markedly blocked by neutralizing antibodies. Although influenza virus could spread via cell-to-cell transmission mode as same as HCV,

there are substantial differences in the virological significance and aim.

Moreover, our results demonstrated that neuraminidase activity played an important role in blocking the super-infection to producer cells by eliminating functional receptor molecules on it and thereby enhanced infection of uninfected neighboring cells around the infected cells. It was suggested that neuraminidase might be involved in the determination of the speed and the direction of the cell-to-cell transmission of wild-type influenza viruses. The new aspect of neuraminidase function has not been described yet.

Our data disclosed the existence of a novel infection mode for influenza viruses. This finding may warn against the biased using of neuraminidase inhibitors, because it was suggested that the effects of neuraminidase inhibitors are not enough to completely block the spread and replication of influenza viruses in microenvironments. This situation probably confers some opportunities to change viral properties and generate mutant variants. As long as this transmission mode exists, we have to search for other targets as antiviral therapeutic strategies against the influenza virus infection.

Future directions

- Biological significances of cell-to-cell transmission of influenza viruses *in vivo*.
- Screening of the specific inhibitor against cell-to-cell transmission.
- Evaluation of the specific inhibitor as the drugs for influenza therapeutics.

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参 考 論 文

Tamiflu-Resistant but HA-Mediated Cell-to-Cell Transmission through Apical Membranes of Cell-Associated Influenza Viruses

Kotaro Mori¹, Takahiro Haruyama^{1,2}, Kyosuke Nagata*

Department of Infection Biology, Faculty of Medicine and Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan

Abstract

The infection of viruses to a neighboring cell is considered to be beneficial in terms of evasion from host anti-virus defense systems. There are two pathways for viral infection to “right next door”: one is the virus transmission through cell-cell fusion by forming syncytium without production of progeny virions, and the other is mediated by virions without virus diffusion, generally designated cell-to-cell transmission. Influenza viruses are believed to be transmitted as *cell-free* virus from infected cells to uninfected cells. Here, we demonstrated that influenza virus can utilize cell-to-cell transmission pathway through apical membranes, by handover of virions on the surface of an infected cell to adjacent host cells. Live cell imaging techniques showed that a recombinant influenza virus, in which the *neuraminidase* gene was replaced with the *green fluorescence protein* gene, spreads from an infected cell to adjacent cells forming infected cell clusters. This type of virus spreading requires HA activation by protease treatment. The cell-to-cell transmission was also blocked by amantadine, which inhibits the acidification of endosomes required for uncoating of influenza virus particles in endosomes, indicating that functional hemagglutinin and endosome acidification by M2 ion channel were essential for the cell-to-cell influenza virus transmission. Furthermore, in the cell-to-cell transmission of influenza virus, progeny virions could remain associated with the surface of infected cell even after budding, for the progeny virions to be passed on to adjacent uninfected cells. The evidence that cell-to-cell transmission occurs in influenza virus lead to the caution that local infection proceeds even when treated with neuraminidase inhibitors.

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* E-mail: knagata@md.tsukuba.ac.jp

These authors contributed equally to this work.

Current address: Central Research Center, AVSS Corporation, Nagasaki, Japan

Introduction

It is generally accepted that viruses, released as *cell-free* virions from an infected cell, transmit to distant cells and tissues. This spreading pathway contributes to wide-ranged diffusion of *cell-free* viruses. However, in this spreading pathway, viruses are exposed to host anti-virus defense systems. In contrast, direct infection to a neighboring cell is considered to be beneficial for the virus in terms of evasion from the host anti-virus defense. There are two typical manners in infection to “right next door”: one is the virus transmission through cell-cell fusion by forming syncytium without production of progeny virions, and the other is mediated by virions without virus diffusion, generally designated cell-to-cell transmission [1,2].

The cell-cell fusion infection pathway is characteristic for a variety of virus such as paramyxoviruses, herpesviruses, some retroviruses, and so on. For example in the case of measles virus belonging to *Paramyxoviridae*, infection is initiated by the interaction of the viral hemagglutinin glycoprotein with host cell surface receptors. The virus penetrates into the cell through membrane

fusion mediated by the interaction of the fusion glycoprotein. In later stages of infection, newly synthesized glycoproteins accumulate at the cell membrane resulting in fusion of the infected cell with neighboring cells by producing syncytia. Thus, viruses can spread from cell to cell without producing *cell-free* virus particles.

The examples of the cell-to-cell transmission are diverse, and these mechanisms are dependent on pairs of viruses and host cells. Vaccinia virus particles bound on the filopodium of an infected cell are repelled toward neighboring uninfected cells by the formation of filopodia using actin filament [3]. The filopodia direct viruses to uninfected cells. Immunotropic viruses including retroviruses utilize an immunological synapse, designed as virological synapses for the cell-to-cell transmission [4–7]. Claudin-1 and occludin, components of tight junction, are involved in hepatitis C virus (HCV) entry through the cell-to-cell transmission [8,9]. The cell-to-cell transmission through tight junction is also observed in other viruses which infect epithelial layers [10,11]. These retroviruses and HCV remain on the surface of an infected cell even after budding. The uninfected cells adjacent to these infected cells can accept or take over viruses from

the infected cell. Thus, the cell-to-cell transmission can be categorized into two manners based on the state of infecting viruses, either *cell-free* or cell-associated virions.

Influenza virus, belonging to the family of *Orthomyxoviridae*, is one of the most serious zoonotic pathogens and causes seasonal epidemics or periodic pandemics among human beings around the world. The viral envelope consists of a lipid bilayer derived from cells that anchors three of viral transmembrane proteins, hemagglutinin (HA), neuraminidase (NA), and matrix protein 2 (M2). Influenza virus infection is initiated by the attachment of HA on virus particles to cell surface receptors containing sialic acids [12]. It has been known that the specific interaction between HA and sialic acid species is one of the determinants of the host range of influenza viruses [13]. Beside its role in the viral attachment, HA is also involved in intracellular fusion between viral envelope and host cell endosome membrane in the endocytotic pathway, by which the virus content is released inside the host cell [14]. The functional maturation of HA is mediated by the cleavage of HA into two disulfide-linked glycopolypeptides, HA1 and HA2 [15], accomplished by trypsin or trypsin-like proteases derived from host cells [16–19]. The membrane fusion is induced by a conformational change in the mature HA, which is triggered at low pH in the endosome, allowing viral ribonucleoprotein complexes to release into the cytoplasm [20,21]. Thus, HA plays a critical role in initiation and progression of influenza virus infection. Influenza virus NA possesses the enzymatic activity that cleaves α -ketosidic linkages between terminal sialic acids and adjacent sugar residues of cellular glycoconjugates [22]. The sialidase activity of NA removes terminal sialic acid residues from HA and NA proteins as well as host cell surface glycoproteins. Since the terminal sialic acid of sialyloligosaccharides is critical for HA binding, the receptor-destroying activity of NA serves to counter the receptor-binding activity of HA. It is quite likely that this activity contributes to prevention of successive superinfection of an infected cell [23]. In the absence of the functional sialidase activity, progeny virions aggregate on the cell surface due to the HA receptor-binding activity and can not be released [24,25]. Thus, NA cleaves sialic acids from the cell surface and facilitates virus release from infected cells. However, it is not clear whether every progeny virion is released as *cell-free* virion to infect the uninfected cells after diffusion into the extracellular environment. Influenza viruses are generally transmitted as *cell-free* viruses from infected to uninfected cell but they may also infect through the cell-to-cell transmission, in particular during local lesion formation.

Here, we examined whether influenza virus transmits from an infected cell to adjacent uninfected cells without virus release. Live cell imaging techniques showed that a recombinant influenza virus, in which the *NA* gene was replaced with the *green fluorescence protein* gene, spreads from an infected cell to adjacent cells forming infected cell clusters. Furthermore, progeny virions remain associated on the surface of infected cell even after budding, and then progeny virions could be passed to adjacent uninfected cells.

Results

Influenza virus can spread in an NA-independent manner to adjacent cells

To examine the transmission pathway of influenza virus, we performed immunofluorescence analyses by using anti-nucleoprotein (NP) polyclonal antibody. Influenza virus can form an infection center even in the presence of oseltamivir, a potent NA inhibitor (commercially known as Tamiflu) [26–28]. Oseltamivir at the concentration of 50 $\mu\text{g/ml}$ completely prevented the release of progeny influenza viruses (Figure 1A). Noted that a large

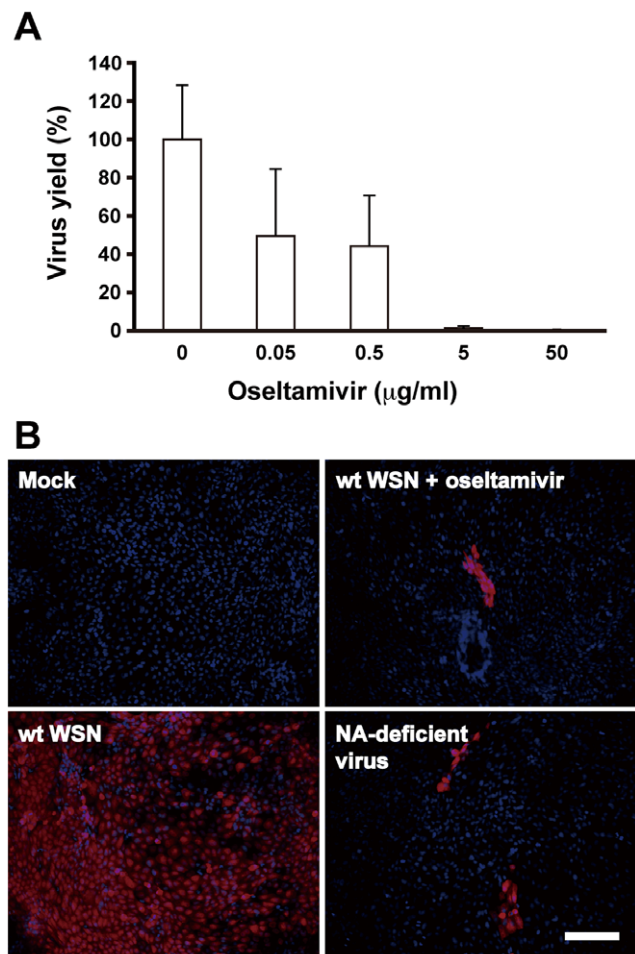


Figure 1. Influenza viruses can spread independent of the NA activity. (A) MDCK cells were infected with influenza virus A/WSN/33 at a multiplicity of infection (MOI) of 0.001 PFU per cell. At 48 hours post infection (hpi), culture supernatant was collected, and then its virus titer was determined by plaque assays. Each result was represented by a value relative to that in the absence of the drug. Error bars indicate standard deviation (s.d.) from 3 independent experiments. (B) Confluent MDCK cells were infected by wild-type influenza virus A/WSN/33 or NA-deficient influenza virus at MOI of 0.0001 in the presence or absence of 50 $\mu\text{g/ml}$ oseltamivir phosphate. NA-deficient influenza virus was generated by reverse genetics as previously described [29]. After incubation at 37°C for 36 hours, immunofluorescence analyses were performed using anti-nucleoprotein (NP) polyclonal antibody and anti-rabbit IgG antibody conjugated to Alexa Fluor 568 (Invitrogen). Scale bar, 100 μm .

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number of single fluorescent foci caused by initial infection markedly expanded and formed cell clusters consisting of 5–10 infected cells in an MDCK cell monolayer (Figures 1B and S1), suggesting influenza virus can spread to some extent in the presence of oseltamivir. To verify that NA is not involved in this spreading, we generated an NA-deficient influenza virus by a reverse genetics method as described previously [29,30]. The NA-deficient influenza virus contains a mutated NA segment, in which the NA coding region including a sialidase catalytic domain was replaced with the *enhanced green fluorescent protein (EGFP)* gene [29]. By this replacement, the NA activity is eliminated from the recombinant influenza virus, and *EGFP* can be utilized as a marker for viral infections. Immunofluorescence analyses demonstrated that the NA-deficient influenza virus also forms infected cell

clusters similarly to those formed by wild-type influenza virus in the presence of oseltamivir (Figure 1B). The fluorescence pattern of NP overlapped with the localization of GFP derived from the *EGFP* gene of the NA-deficient influenza virus (Figure S2). Thus, NA-deficient influenza virus can be used to investigate the NA-independent infection pathway of influenza virus.

Next, we performed live cell imaging analyses to directly observe the infection time course of the NA-deficient influenza virus. The GFP fluorescence derived from the NA-deficient influenza virus first appeared in a single cell on an MDCK cell monolayer at 24 hours post infection. The virus started to spread from an infected cell to adjacent cells in 5–6 hours after the first appearance of a GFP-positive cell (Figure 2 and Video S1). The spreading rate was clearly faster than the rate of cell divisions. The mean doubling time of uninfected MDCK cells was 20–24 hours under the condition employed here, and it is expected that the proliferation speed would be much slowly because infected MDCK cells were maintained in the serum-free medium and formed cell monolayer at the high cell density. These suggest that NA-deficient influenza viruses may infect adjacent cells through the cell-to-cell transmission mechanism without apparent production of *cell-free* virions.

Cell-to-cell transmission pathway of influenza viruses is less sensitive to neutralizing antibody

The cell-to-cell virus transmission pathway could be interpreted as one of viral evolving strategies to avoid neutralizing antibody responses [2,31,32]. Therefore, we examined the effect of neutralizing antibody on NA-deficient influenza virus. A polyclonal antibody with the neutralizing activity against influenza virus particles inhibited infection of *cell-free* viruses to less than 50% at the concentration of 0.03%, although the cell cluster formation was observed at the concentration less than 0.01%. On the other hand, the NA-independent transmission of the NA-deficient

influenza virus was blocked only when neutralizing antibody was present at the concentration of 0.3% (Figure 3). These results indicated that the NA-independent transmission of influenza viruses is less sensitive to the neutralizing antibody.

NA-independent transmission of influenza virus is HA-dependent

Next, to investigate the mechanism of NA-independent transmission of influenza virus, we examined whether HA is involved in this transmission. In the absence of the NA activity, virus spreading from an infected cell to adjacent cells was dramatically suppressed by omission of trypsin, essential for maturation of HA, from the experimental condition (Figure 4A). The GFP fluorescence derived from NA-deficient influenza virus appeared in a single cell at 24 hours post infection. However, this virus did not spread, but rather disappeared during subsequent 24 hours (Video S2). These observations indicate that the NA-independent cell-to-cell transmission of influenza virus is dependent on HA maturation mediated by trypsin, as is the case for the general *cell-free* transmission of this virus.

To clarify whether virus particles or viral RNP complexes are transmitted to adjacent cells, we examined the effect of amantadine on the cell-to-cell transmission of influenza virus. Amantadine inhibits the early step of uncoating of influenza virus RNP from virion in endosomes [33,34]. For this study, other influenza virus strain, influenza virus A/Udorn/72, was used instead of influenza virus A/WSN/33 because influenza virus A/WSN/33 is highly resistant to amantadine [35]. We confirmed that influenza virus A/Udorn/72 is sensitive to oseltamivir (Figure S3) and could also spread via cell-to-cell transmission independent of the NA activity as did for influenza virus A/WSN/33 (Figures 1B and 4B). In the case of a single administration of amantadine, fluorescent foci derived from infected cells scattered, and the number of single foci was greatly decreased compared

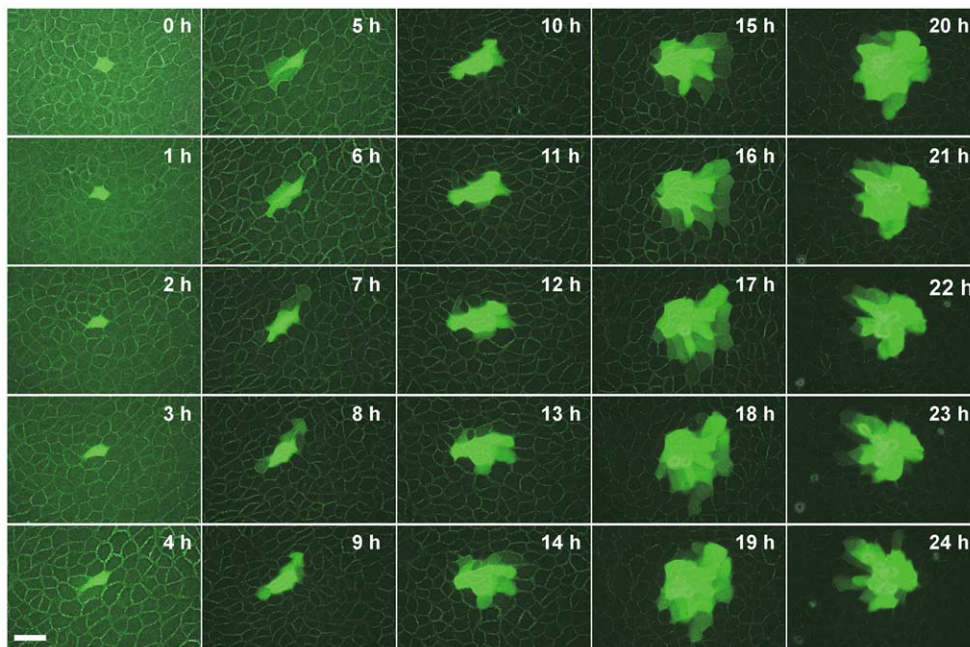


Figure 2. NA-deficient influenza virus spreads through cell-to-cell transmission. Confluent MDCK cells were infected with the NA-deficient influenza virus at MOI of 0.0001. After incubation at 37°C for 24 hours, a single GFP-positive cell, in which the recombinant virus replicated, was found at 1 hour after starting monitoring, and then this cell and its neighborhood were traced during the period from 24 hpi to 48 hpi at interval of 1 hour. Scale bar, 50 μ m.

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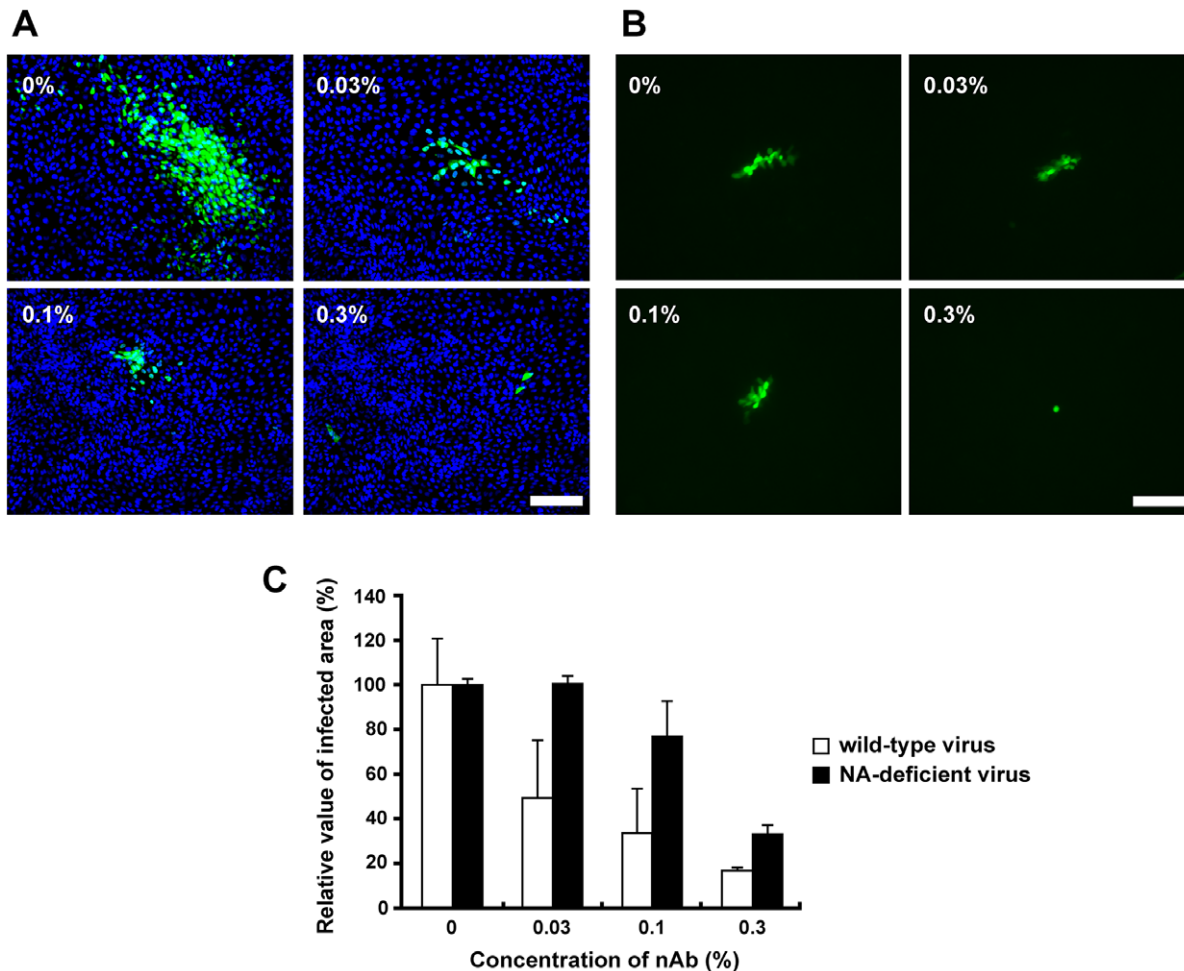


Figure 3. The cell-to-cell transmission of the NA-deficient influenza virus is less sensitive to the neutralizing antibody. (A) Infection of the wild-type and (B) NA-deficient influenza virus were performed in the presence or absence of antiserum containing neutralizing antibodies. Immunofluorescence analyses were performed with cells infected with wild-type influenza virus at 18 hpi using anti-NP antibody and anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Invitrogen). GFP fluorescence derived from the recombinant virus was observed at 36 hpi. Scale bar, 100 μ m. (C) The level of viral spreading was indicated in the graph by measuring NP and GFP derived from wild-type and NA-deficient virus, respectively. Five different microscope fields were taken randomly, and then the intensity of green color was analyzed with ImageJ NIH image processing software. Each result was represented by a value relative to that in the absence of neutralizing antibodies. Error bars indicate s.d. from 3 independent experiments.
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with that in the absence of the drugs. In contrast, a single administration of oseltamivir, fluorescent foci formed some clusters and expanded in a time-dependent manner (Figure 4B). This dissimilarity of inhibitory manner was caused by the difference of the sites of action between amantadine and oseltamivir. Amantadine inhibits the replication of influenza A virus by preventing the translocation of vRNP complexes from endosomes to the cytoplasm, whereas oseltamivir has no effects on viral replication itself but inhibits the release of *cell-free* virions from infected host cells. We investigated the inhibitory effect of amantadine on the cell-to-cell transmission of influenza viruses. The formation of infected cell clusters was observed with co-administration of amantadine and oseltamivir, as well as with a single administration of oseltamivir (Figure 4B). However, the quantitative analysis revealed that the size of infected cell clusters with the co-administration were decreased as compared to that with oseltamivir alone (Figure 4C). These observations indicated that the NA activity-independent cell-to-cell transmission of influenza virus was susceptible to the inhibitory effect of amantadine,

suggesting that the cell-to-cell transmission undergoes through endocytosis but vRNP complex itself is not incorporated in the infected cells by adjacent cells.

Cell-to-cell transmission occurs on the apical cell membrane

The virus transmission undergoes from infected to uninfected cells through either basolateral [36–38] or apical [39–42] sides. In the case of influenza virus, *cell-free* progeny virions are released only from the apical surface of polarized epithelial cells [43]. This releasing polarity is achieved by directed transport of viral membrane proteins to the apical plasma membrane [44]. Indeed, that HA and NA glycoproteins are associated with lipid rafts, and the raft association has been implicated in apical transport [45,46].

To determine whether or not the cell-to-cell transmission of the NA-deficient influenza virus occurs on the apical surface, we performed transwell assays in the presence of the neutralizing antibody to influenza A viruses. The neutralizing antibody was added to infected MDCK cell monolayer from apical or

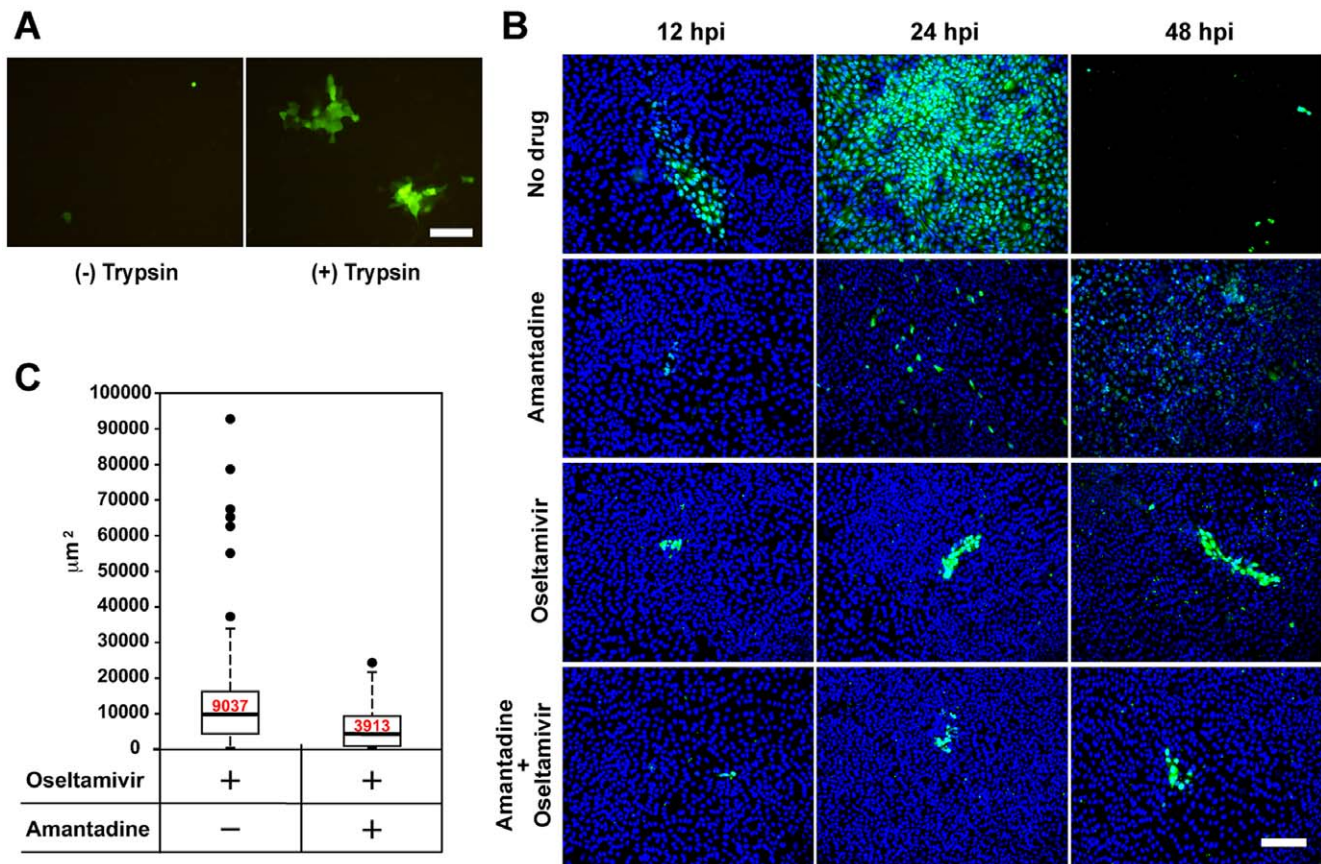


Figure 4. The cell-to-cell transmission of the NA-deficient influenza virus requires functional HA. (A) Confluent MDCK cells were infected with the NA-deficient influenza virus at MOI of 0.0001 in the presence or absence of 1 $\mu\text{g}/\text{ml}$ trypsin. GFP fluorescence derived from the recombinant virus was observed at 36 hpi. Scale bar, 100 μm . (B) MDCK cells were infected with influenza virus A/Udorn/72 at moi of 0.0001 in the presence or absence of 50 μM amantadine or 50 $\mu\text{g}/\text{ml}$ oseltamivir phosphate. Amantadine at the concentration of 50 μM almost completely inhibited the production of progeny virions (data not shown). After incubation for 12, 24, and 48 h, immunofluorescence analyses were performed using anti-NP antibody and anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Invitrogen). Viral NP and nuclear DAPI staining are shown in green and blue, respectively. Scale bar, 100 μm . (C) Median sizes of clusters were shown as box plots summarizing sizes of 60 individual infectious foci formed in the presence of oseltamivir alone, or both oseltamivir and amantadine. Immunofluorescence analyses were performed as described in (B) at 24 hpi. Boxes enclose the lower and upper quartiles; thick horizontal lines represent the median; dashed lines indicate the extreme values; and black dots are outliers of individual infectious foci. The size of infectious foci was measured with AxioVision Release 4.7.2 imaging software (Carl Zeiss). Median sizes shown in red letters were clearly different from each other ($p < 0.01$). doi:10.1371/journal.pone.0028178.g004

basolateral side, and the inhibitory effect on the spread of GFP fluorescence derived from the recombinant virus was examined. Addition of high concentrations of the neutralizing antibody from the apical side blocked the cell-to-cell transmission of the NA-deficient influenza virus, whereas the addition from the basolateral side had no effect (Figure 5). These observations indicated that the polarity in the influenza virus budding in the cell-to-cell transmission pathway is apical.

Influenza viruses can not re-infect previously infected cells

Previous report showed that influenza viruses were refractory to superinfection with a second cell-free virus [23]. In the case of the cell-to-cell transmission of influenza virus in the presence of oseltamivir, it is possible that a progeny virion is temporarily bridged by HA between an infected cell and adjacent uninfected cells, since viruses can not be released from infected cell surface due to the inhibition of the NA activity by oseltamivir. The cell-associated progeny virion may have an opportunity to re-infect the previously infected cell, compared to a cell-free progeny virion in

the general spreading. Thus, we examined whether influenza viruses can infect the cell which had already been infected, using *t53* mutant and wild-type influenza virus A/WSN/33. *t53* virus has a substitution mutation from U to C at the nucleotide position of 701 in the PA gene. This substitution introduces an amino acid change from wild-type Leu 226 to Pro 226 and gives a defect in the viral genome replication process [47,48]. At first, cells were infected with *t53* virus at moi of 10, and after incubation for 0, 2, 4, 6, and 8 hours, cells were superinfected with wild-type virus at moi of 10. The amount of segment 3 viral RNA (vRNA) encoding PA was determined quantitatively by RT-PCR. Then, using a mutated primer for PCR, we could introduce a *Stu* I site only in the PCR products derived from the wild-type sequence (Figure 6A). Thus, DNA fragments amplified from the wild-type and *t53* could be distinguished by *Stu* I digestion. The digested DNA fragments containing 220 and 199 base pairs derived from *t53* and wild-type, respectively, were separated through PAGE. After 6 hours or later post infection, re-infection with the second challenging virus hardly occurs in the absence of oseltamivir. However, in the presence of oseltamivir, appearance of wild-type fragment suggests that the re-infection had occurred (Figure 6B). The result indicates

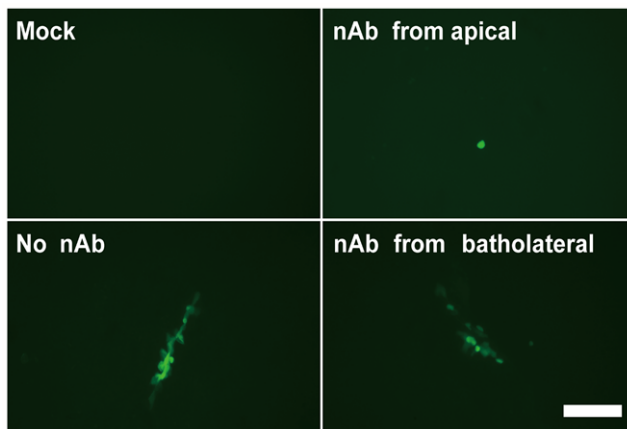


Figure 5. The cell-to-cell transmission of the NA-deficient influenza virus occurs the apical cell surface. Confluent MDCK cells were prepared in transwell inserts and infected with the NA-deficient influenza virus at MOI of 0.0001 in the presence or absence of 0.3% (v/v) antiserum containing neutralizing antibodies (nAb) to influenza A virus. After virus adsorption, the antiserum was added from apical or basolateral side. GFP fluorescence derived from the recombinant virus was observed at 36 hpi. The antiserum added from the apical side could markedly block the cell-to-cell transmission of the NA-deficient influenza virus, whereas the antiserum added from the basolateral side could not. Scale bar, 100 μ m. doi:10.1371/journal.pone.0028178.g005

that progeny virus particles remain on the surface of infected cell even after budding, and can infect the cell previously infected, as well as uninfected cells adjacent to the infected cell, when oseltamivir is present.

Discussion

With the except for the virus which spreads through the cell-cell fusion transmission, virus infection is initiated by the binding of *cell-free* virions to their host cells. Recently, the virus transmission mechanism from an infected cell to adjacent cells without virus diffusion into the extracellular environment is highlighted from the aspect of its significance in virus spreading in the presence of antibodies [1,2]. This antibody-insensitive pathway is often called cell-to-cell transmission [2]. The cell-to-cell transmission may be categorized into two pathways, *i.e.*, transmission of *cell-free* virions to adjacent uninfected cells, and transmission of progeny virions associated on the surface of an infected cell even after budding through narrow synaptic space between an infected cell and adjacent uninfected cells. As an example of the former mechanism, *cell-free* vaccinia virus particles associated with the filopodium of an infected cell are repelled toward neighboring uninfected cells by inducing the formation of actin filament [3]. Several cases have been reported for the latter mechanism: Immunotropic viruses including retroviruses utilize the immunological synapses [4–7]. Immune cells are not constitutively polarized, but contain the machinery that directs their secretory apparatus towards a cell that is involved in an immunological synapse. This machinery can be subverted by retroviruses containing human immunodeficiency virus (HIV). An HIV-infected cell can polarize viral budding towards a target cell expressing receptor through a structure called a virological synapse. Virions bud from an infected cell into a synaptic cleft, from which they fuse with the target-cell plasma membrane [49–52]. The progeny virions of HCV are trapped between infected and uninfected cell membranes at the tight junction. Using Claudin-1 known as a component of the tight

junction and one of the entry factors of HCV [8], virions fuse with and penetrate uninfected target cells [31]. Therefore, HCV may acquire the ability to spread within polarized liver epithelium. Thus, the cell-to-cell transmission certainly plays significant roles for the dissemination of several enveloped viruses. However, the cell-to-cell transmission of influenza virus has not been discussed well. Here, we have shown that influenza virus spreads by forming infected cell clusters even in the presence of an NA inhibitor. Live cell imaging clearly showed that influenza virus lacking the NA activity spreads from an infected cell to adjacent cells through the cell-to-cell transmission mechanism (Figure 2). This was also the case for wild-type influenza virus during early phases of infection (Figure 4B). In the cell-to-cell transmission of influenza virus, progeny virions could remain associated with the surface of infected cell even after budding, and then these progeny virions can be passed on to adjacent uninfected cells.

We showed that the cell-to-cell transmission of the NA-deficient influenza virus depends on functional HA. The viral spreading was dramatically suppressed without HA activation by trypsin treatment (Figure 4A). Moreover, the cell-to-cell transmission was also blocked by amantadine, which inhibits the acidification of endosomes required for uncoating of influenza virus particles in endosomes [33,34]. These findings indicate that functional HA and endosome acidification by M2 ion channel are required for the cell-to-cell influenza virus transmission, thereby allowing viruses to enter the adjacent cells through the endocytotic pathway (Figure 4).

Our findings showed that the NA-deficient influenza virus is not diffused into the extracellular environment. The viral spreading in the absence of oseltamivir appears to be much faster compared to the viral spreading in the presence of the drug, suggesting that NA could be involved in determination of spreading speed (Figure 4B). The NA activity prevented progeny virions from entering cells which virus came from (Figure 6), implying that progeny virus particles should be transmitted to adjacent uninfected cells. The cell-to-cell transmission started in early phase of infection, and the virus spread through diffusion of *cell-free* viruses (Figure 4B). Indeed, it was reported that the cell-to-cell transmission is a rapid spreading pathway in the case of vaccinia virus [3]. Vaccinia virus induces a blocking mechanism of superinfection and thereby infects to adjacent uninfected cells efficiently. In early phases of vaccinia virus infection, viral proteins A33 and A36 are expressed at the infected cell surface. Once *cell-free* virus particles contact the filopodium, the A33/A36 complex induces the formation of actin filament, which causes this superinfected virion to be repelled toward uninfected cells [3]. Influenza viruses can re-infect the cells previously infected in the presence of oseltamivir (Figure 6), suggesting that a progeny virion may be bridged by HA between infected and adjacent uninfected cells temporarily. Thus, in the case of the cell-to-cell transmission of influenza virus, we propose that progeny virions associated with the surface of infected cells even after budding are directed to adjacent uninfected cells. The cell-to-cell transmission mechanism of influenza virus is distinctly different from that of vaccinia virus in the infecting virus status: Infected cell-associated virions and *cell-free* virions are involved in the cell-to-cell transmission of influenza virus and vaccinia virus, respectively. The strategy for influenza virus appears to be similar to that for HCV. HCV progeny virions budded from an infected cell are trapped between infected and uninfected adjacent cell membranes at the tight junction. HCV virions then, enter into adjacent cells through endocytosis and low pH-dependent membrane fusion using Claudin-1 [8]. The cell-to-cell transmission of influenza virus also required functional HA and endosome acidification by M2 ion channel. However, it has not been reported that HCV has a gene encoding a receptor destroying

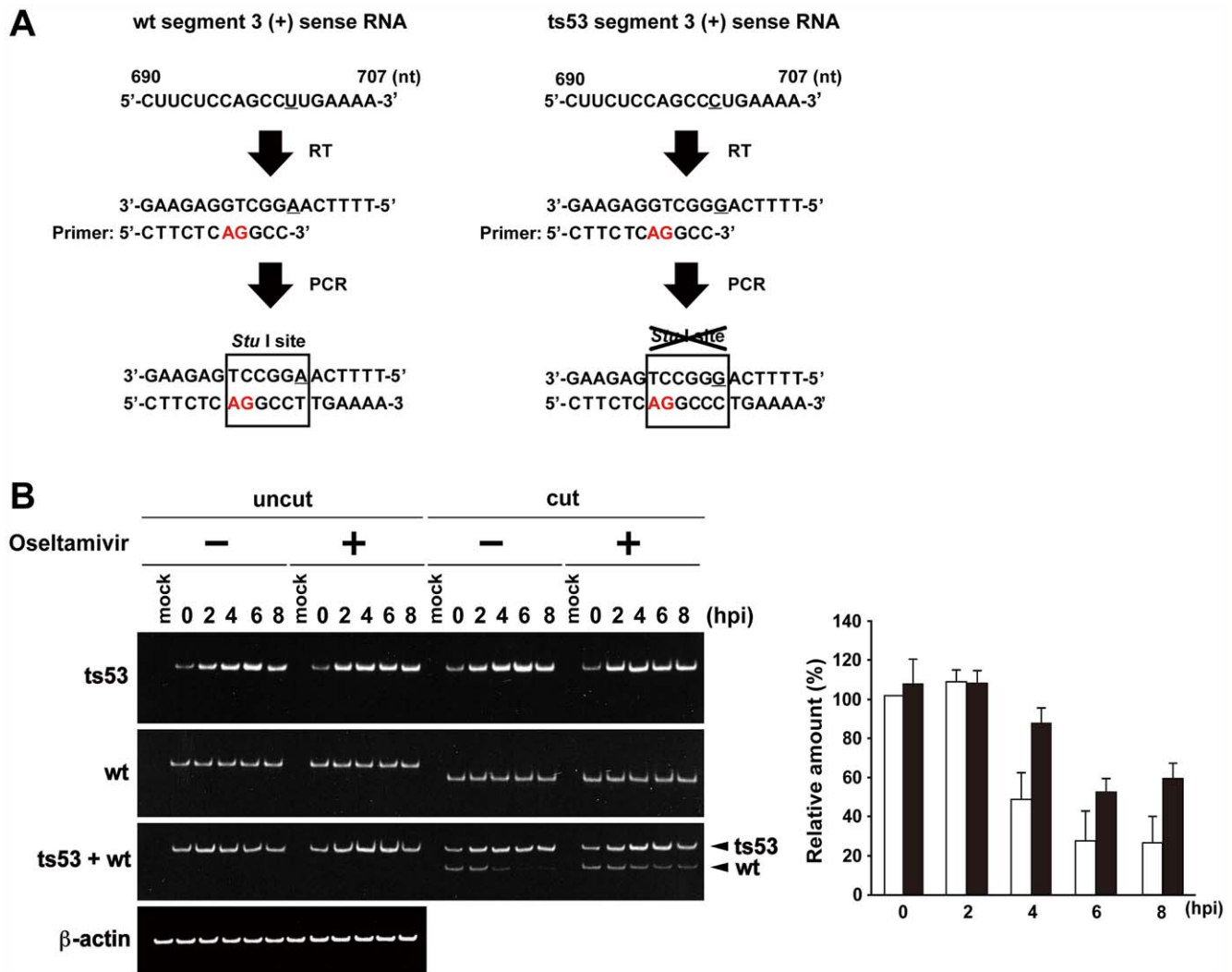


Figure 6. Influenza viruses can not re-infect previously infected cells. (A) A method for determination of the amount of segment 3 genome derived from ts53 and wild-type. Total RNA was reverse-transcribed with the primer PA-895-rev, which is complementary to the segment 3 positive-sense RNA. The cDNA was amplified by PCR using primers, PA-895-rev and PA-695-cut partially corresponding to segment 3 positive sense RNA between the nucleotide sequence positions 678 to 700 except for 696 and 697, which are shown in red letters. Since segment 3 of ts53 has a substitution mutation from U to C at the nucleotide position of 701, the PCR product derived from wild-type could be digested by *Stu* I but not that from ts53. Then, PCR products were digested with *Stu* I and separated through 8% PAGE. (B) Detection of the genome of the segment 3 derived from ts53 or wild-type. At 3 hours post superinfection of wild-type virus, total RNA was extracted, and semi-quantitative RT-PCR was performed. Subsequently, the amplified DNA products were digested with *Stu* I and separated through 8% PAGE. Large and small fragments derived from ts53 and wild-type viruses were 220 and 199 base pairs, respectively. The relative amount of wild-type segment 3 to that at 0 hour in the absence of oseltamivir phosphate was shown in the graph. Error bars indicate S.D. from 3 independent experiments. White bar, in the absence of oseltamivir phosphate; black bar, in the presence of oseltamivir phosphate.

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enzyme similar to NA of influenza virus. We speculated that HCV progeny particles are bridged between infected and adjacent uninfected cells temporarily like influenza virus in the presence of oseltamivir. Progeny influenza virus particles could be transmitted to adjacent uninfected cells efficiently in the presence of the NA activity, suggesting that the cell-to-cell transmission of influenza virus is more strategic than that of HCV.

Our findings raise an interesting question as to what is the biological significance of cell-to-cell transmission for influenza virus infection *in vivo*. Until now, it had been believed that influenza virus was released from infected cells as *cell-free* virions and then spread from cell to cell as well as from organism to organism. The transmission mode by *cell-free* virions undergoes the extremely high-speed of its diffusion and causes epidemic or pandemic infection.

The tropism in an infected animal body is generally restricted to respiratory tract or lung and its periphery, and the requirement of a trypsin-like protease has been generally described for the reason of the restriction. It is possible that the cell-to-cell transmission mode may play a significant role for the virus spreading inside of organism, although *cell-free* influenza virions are causative of high-speed spreading. At the least, the limited but distinct level of infection followed by replication could provide some opportunity to generate influenza virus variants. It is an open question whether the cell-to-cell transmission mode is involved in the pathogenesis caused by influenza virus infection *in vivo*.

The existence of cell-to-cell transmission pathway gives a caution when NA inhibitors are used, because NA inhibitors may not be sufficient to completely block the spread of influenza

virus in local microenvironments. Since this cell-to-cell transmission pathway exists, development of antiviral therapeutic strategies in addition to NA inhibitors is highly recommended.

Materials and Methods

Cells and viruses

Madin-Darby canine kidney (MDCK) cells were kindly gifted by A. Ishihama (Hosei University), and maintained in minimal essential medium (MEM) (Nissui) containing 10% fetal bovine serum. Human embryonic kidney 293T cells were kindly gifted by Y. Kawaoka (University of Tokyo), and maintained in Dulbecco modified Eagle medium (DMEM) (Nissui) supplemented with 10% fetal bovine serum. Influenza virus A/Udorn/72 was grown in allantoic sacs of 11 day-old embryonated eggs (MIYAKE HATCHERY). Wild-type influenza virus A/WSN/33 and *t*53 mutant were used after single-plaque isolation. MDCK cells were infected with influenza virus A/WSN/33 or *t*53 at a multiplicity of infection (MOI) of 0.1 PFU/cell, and incubated at 37°C and 34°C, respectively. After incubation for 24 h, the culture fluid was harvested and centrifuged at 1,700× *g* for 10 min. The virus suspension was stored at −80°C until use.

Antibodies

The production of rabbit polyclonal anti-NP antibody was described previously [53], and this antibody was used as a primary antibody for indirect immunofluorescence assay. A goat anti-rabbit IgG antibody conjugated to Alexa Fluor 488 or Alexa Fluor 568 was purchased from Invitrogen and used as a secondary antibody for indirect immunofluorescence assay. A polyclonal antibody against influenza A virus was obtained from 2-month-old female rabbit immunized with 250 µg of purified virions of influenza virus strain A/Puerto Rico/8/34 [54]. The generation of antibodies was boosted three times and used as neutralizing antibodies to block the influenza virus infection.

Determination of the inhibition effect of oseltamivir on virus production

MDCK cells were infected with influenza virus A/WSN/33 at a multiplicity of infection (MOI) of 0.001 PFU per cell. After virus adsorption at 37°C for 1 hour, the cells were washed with serum-free MEM and incubated at 37°C with maintenance medium (MEM containing vitamins and 0.1% BSA) containing oseltamivir. At 48 hours post infection (hpi), culture supernatant was collected, and then its viral titer was determined by plaque assays.

Generation of neuraminidase (NA)-deficient viruses

An NA-deficient influenza virus possessing the terminal sequences of NA segment but lacking the NA coding region, which was replaced with *enhanced green fluorescent protein (EGFP)* gene, was generated by reverse genetics as described previously [29,30]. For reverse genetics, we used plasmids containing cDNAs of the influenza virus A/WSN/33 viral genome under the control of the human RNA polymerase I promoter (referred to as Pol I plasmids). Briefly, 293T cells were transfected with seven Pol I plasmids for production of all vRNA segments of influenza virus A/WSN/33 and one for the mutant NA vRNA segment containing *EGFP* ORF, together with protein expression vectors for PB2, PB1, PA, and NP controlled by the chicken β-actin promoter (pCAGGS). TransIT-293 (Mirus) was used for transfection. At 24 hours post transfection, recombinant viruses were harvested from the cell surface using bacterial NA derived from *Clostridium perfringens* (sigma). MDCK cells were infected with harvested recombinant viruses treated with *N*-tosyl-L-phenyl-

alanine chloromethyl ketone (TPCK)-trypsin (1 µg/ml). After confirmation of GFP fluorescence derived from amplified recombinant virus genomes at 48 hours after infection, the recombinant viruses on the cell surface were collected using bacterial NA. The viral titer of recombinant viruses was determined by counting the number of infected foci using a fluorescence microscopy (Carl Zeiss).

Indirect immunofluorescence assay

Cells on coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with 0.2% NP-40 in PBS. The coverslips were soaked in 1% bovine serum albumin in PBS, and then incubated at room temperature for 1 hour with a primary antibody. After being washed twice with PBS, the coverslips were incubated at room temperature for 1 hour with a secondary antibody. The coverslips were then incubated at room temperature for 5 min with 3 µM 4',6'-diamidino-2-phenylindole (DAPI) and finally mounted on glass plates, and cells were observed under the fluorescence microscope.

Live cell imaging analyses

Living cells were analyzed using BioStation ID system (GE Healthcare). Confluent MDCK cells were infected with the NA-deficient influenza virus at the multiplicity of infection (MOI) of 0.0001 in the presence or absence of 1 µg/ml TPCK-trypsin. At 24 hours post infection, culture dishes containing infected cells were set into the chamber of BioStation ID system, which was maintained at 37°C under 5% CO₂ and 95% humidity. Then, images were acquired during next 24 hours at interval with 1 hour. The excitation wavelength was controlled by a manual filter wheel equipped with filters suitable for enhanced green fluorescence protein (EGFP).

Transwell assay

Confluent MDCK cell monolayer was prepared on transwell inserts (BD Falcon, pore size 0.4 µm) and infected with the NA-deficient influenza virus at MOI of 0.0001. After virus adsorption at 37°C for 1 hour, the cell monolayer was washed with serum-free MEM, and maintenance medium was added into both sides within the transwells. The neutralizing antibody to influenza A virus was added into the inside or the outside of transwell inserts with the maintenance medium. Subsequently, cells were incubated at 37°C for 36 hours followed by analyses using the fluorescence microscopy.

RT-PCR

*t*53 virus has a substitution mutation from U to C at the nucleotide position of 701 in the *PA* gene. This substitution introduces an amino acid change from wild-type Leu 226 to Pro 226 and gives a defect in the viral genome replication process [48]. However, under the permissive temperature, the level of viral genome replication is no difference between wild-type and *t*53 [47]. To discriminate the genome of wild-type and that of *t*53, total RNA was reverse-transcribed by reverse transcriptase (TOYOBO) with PA-895-rev (5'-TTAATTTTAAGGCATC-CATCAGCAGG-3'), which is complementary to the segment 3 positive sense RNA. The cDNA was amplified by PCR using primers, PA-895-rev and PA-695-cut (5'-TCTCCCGCCA-AACTTCTCAGGCC-3') partially corresponding to segment 3 positive sense RNA between nucleotide sequence positions 678 to 700 except for nucleotide positions 696 and 697. Since segment 3 of *t*53 has a substitution mutation from U to C at the nucleotide position of 701, the PCR product derived from wild-type was digested by *Sfu* I but not that from *t*53. After PCR reactions, PCR

products were digested with *Stu* I and separated through PAGE. Large and small fragments derived from *t53* and wild-type viruses were 220 and 199 base pairs, respectively. DNA was stained with GelRed (BIOTIUM) and visualized by UV illumination.

Supporting Information

Figure S1 Formation of cell cluster caused by initial infection. MDCK cells were infected with influenza virus A/WSN/33 at moi of 0.0003 in the presence or absence of 50 µg/ml oseltamivir phosphate. After incubation for 8 and 24 h, immunofluorescence analyses were performed using anti-NP antibody and anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Invitrogen). Nuclear DAPI and viral NP staining patterns are shown in blue and green, respectively. Enlarged views are shown in red borders. Scale bar, 100 µm. (TIF)

Figure S2 The expression of GFP derived from NA-deficient influenza virus overlapped with the localization of NP. MDCK cells were infected with NA-deficient influenza viruses at MOI of 0.0001. After incubation at 37°C for 48 hours, immunofluorescence analyses were performed using anti-NP antibody. Scale bar, 100 µm. (TIF)

Figure S3 Influenza virus A/Udorn/72 was sensitive to oseltamivir. MDCK cells were infected with influenza virus A/Udorn/72 at a MOI of 0.001 PFU per cell. At 36 hpi, the culture supernatant was collected, and then its virus titer was determined by plaque assays. Each result was represented by a value relative to that in the absence of the drug. Error bars indicate s.d. from 3 independent experiments. (TIF)

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Video S1 NA-deficient influenza virus spreads through cell-to-cell transmission. Confluent MDCK cells were infected with NA-deficient influenza virus at MOI of 0.0001 in the presence of trypsin. After incubation at 37°C for 24 hours, a single GFP-positive cell and its vicinity were traced it during the period from 24 hpi to 48 hpi at interval of 1 hour. Live cell imaging data analyses was performed by Biostation ID (GE healthcare). Scale bar, 50 µm. (MOV)

Video S2 NA-deficient influenza virus does not spread in the absence of trypsin. Confluent MDCK cells were infected with the NA-deficient influenza virus at MOI of 0.0001 in the absence of trypsin. After incubation at 37°C for 24 hours, a single GFP-positive cell was detected, and then this cell and neighborhood cells was traced during the period from 24 hpi to 48 hpi at interval of 1 hour. Live cell imaging data analyses were performed by Biostation ID (GE healthcare). Scale bar, 50 µm. (MOV)

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Author Contributions

Conceived and designed the experiments: KM TH KN. Performed the experiments: KM TH. Analyzed the data: KM TH KN. Contributed reagents/materials/analysis tools: TH. Wrote the paper: KM TH KN.

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Anti-influenza virus activity of *Ginkgo biloba* leaf extracts

Takahiro Haruyama · Kyosuke Nagata

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Abstract We examined the influence of *Ginkgo biloba* leaf extract (EGb) on the infectivity of influenza viruses in Madin–Darby canine kidney (MDCK) cells. Plaque assays demonstrated that multiplication of influenza viruses after adsorption to host cells was not affected in the agarose overlay containing EGb. However, when the viruses were treated with EGb before exposure to cells, their infectivity was markedly reduced. In contrast, the inhibitory effect was not observed when MDCK cells were treated with EGb before infection with influenza viruses. Hemagglutination inhibition assays revealed that EGb interferes with the interaction between influenza viruses and erythrocytes. The inhibitory effect of EGb was observed against influenza A (H1N1 and H3N2) and influenza B viruses. These results suggest that EGb contains an anti-influenza virus substance(s) that directly affects influenza virus particles and disrupts the function of hemagglutinin in adsorption to host cells. In addition to the finding of the anti-influenza virus activity of EGb, our results demonstrated interesting and important insights into the screening system for anti-influenza virus activity. In general, the plaque assay using drug-containing agarose overlays is one of the most reliable methods for detection of antiviral activity. However, our results showed that EGb had no effects either on the number of plaques or on their sizes in the plaque assay.

These findings suggest the existence of inhibitory activities against the influenza virus that were overlooked in past studies.

Keywords Antiviral effect · *Ginkgo biloba* leaf extract · Hemagglutination · Influenza virus

Introduction

Influenza viruses, members of the *Orthomyxoviridae* family, cause epidemics in the human population every year despite the availability of effective vaccines. In a severe pandemic year, millions of people die from the infection. Influenza viruses are classified on the basis of the antigenic properties of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Sixteen HA subtypes (H1–H16) and nine NA subtypes (N1–N9) have so far been defined. Influenza virus infection is initiated by the interaction between HA and sialic acid moieties of glycoconjugates on host cells [16].

Several synthetic drugs such as amantadine and rimantadine (M2 ion channel inhibitors) and oseltamivir and zanamivir (NA inhibitors) have been available for decades, but all have side effects and thus somewhat limited usefulness [6, 11]. Therefore, novel substances and approaches are needed to control and prevent this viral disease. Various natural products have distinct anti-influenza virus activities [14]. We have demonstrated that a high-molecular-weight lignin-related fraction extracted from cones of *Pinus parviflora* Siebold et Zucc. suppresses the multiplication of influenza viruses by preventing viral RNA synthesis [9, 15]. We also reported that *Sanicula europaea* L. leaf extract contains an anti-influenza virus substance(s) that selectively inhibits influenza A viruses, but

T. Haruyama · K. Nagata (✉)
Department of Infection Biology, Faculty of Medicine and
Graduate School of Comprehensive Human Sciences, University
of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan
e-mail: knagata@md.tsukuba.ac.jp

Present Address:
T. Haruyama
Research Center, AVSS Corporation, 1-22 Wakaba-machi,
Nagasaki 852-8137, Japan

not influenza B viruses [13]. Studies on the anti-influenza virus activity of natural products have dramatically increased over the past several years [14].

Ginkgo biloba leaf extract (EGb) is a potential phyto-medicine with various pharmacologic effects: in particular, anticoagulant, vasodilator, and anti-inflammatory effects [17]. In many countries, EGb and similar products are prescribed as therapeutic agents for cerebral or peripheral vascular inefficiency and for cognitive impairments associated with aging [2, 3]. Unlike other herbal drugs, however, EGb has hardly been tested for its anti-influenza virus activity. In the present study, we examined the inhibitory effect of EGb on influenza viruses.

Materials and methods

Reagents

The powder of *Ginkgo biloba* leaf extract was prepared by Mitsubishi Paper Mills Co., Ltd., Japan. In brief, the dried *Ginkgo biloba* leaves were finely ground and then extracted with water containing alcohol. After removing the residue, the extracts were concentrated under reduced pressure. The concentrate was then filtered and treated with adsorption resin to eliminate the impurities. Finally, the extracts were concentrated under reduced pressure again and then dried to use as powder. The powder of *Ginkgo biloba* leaf extract was dissolved in DMSO at a concentration of 100 mg/ml and stored at -30°C until use.

As main active ingredients, it is known that the extract contains not only flavonoids such as kaempferol, quercetin and isorhamnetin but also terpene lactones such as bilobalide, ginkgolide A, B, C and J as specific components derived from *Ginkgo biloba* leaves [1, 4, 5].

Cells and viruses

Madin–Darby canine kidney (MDCK) cells were maintained in Eagle’s minimum essential medium (MEM) at 37°C , in a 5 % CO_2 atmosphere, supplemented with 10 % fetal bovine serum, 0.03 % L-glutamine, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin.

Influenza A/PR/8/34 (H1N1), A/Udorn/72 (H3N2), and B/Lee/40 viruses were grown at 35.5°C for 48 h in allantoic sacs of 11-day-old embryonated eggs (Miyake Hatchery), and then the infected allantoic fluid was collected and stored at -80°C until use.

Neutral red assay

The neutral red assay is based on incorporation of neutral red into lysosomes in living cells. To determine the effect

of EGb on cell viability, MDCK cells (3.5×10^4 cells/well) were seeded into 24-well tissue culture plates and kept at 37°C overnight. After removal of the culture medium, 0.4 ml of MEM containing various concentrations of EGb or DMSO was added to each well of the plates. After incubation for 24 h at 37°C , 0.2 ml of neutral red solution (0.15 mg/ml) was added to each well. After incubation at 37°C for 3 h, wells were washed with 0.2 ml of a fixative (1 % formalin and 1 % CaCl_2). To extract the dye, 0.2 ml of 1 % acetic acid in 50 % ethanol was added to each well. After incubation at room temperature for 20 min, the amount of neutral red in each well was determined by measuring absorbance at 550 nm using a spectrometer. Results were represented as the cell number that was calculated from the standard curve of cell numbers. Furthermore, to determine the effect of EGb on the cell growth, MDCK cells (2.0×10^4 cells/well) were seeded into 24-well tissue culture plates and kept at 37°C overnight. After removal of the medium, 0.4 ml of MEM containing 0, 10 and 100 $\mu\text{g}/\text{ml}$ of EGb were added to each well. As control groups, DMSO was added to each well at final concentrations of 0.01 or 0.1 %. After incubation at 37°C for 0, 24, 48 and 72 h, viable cells were determined with the neutral red assay as described above.

Treatment of viruses and cells by EGb

For pre-treatment of viruses by EGb, influenza A/PR/8/34 virus (500 pfu/ml) was mixed with EGb at several concentrations, incubated at room temperature for 10 min, and then subjected to the plaque formation assay. For post-treatment by EGb, MDCK cells infected with influenza viruses were overlaid with 0.8 % agarose containing EGb at several concentrations in the plaque formation assay. To investigate the direct effect of EGb on host cells, MDCK cells were exposed to EGb at several concentrations and incubated at 37°C for 1 h. After removing the medium containing EGb, MDCK cells were infected with influenza viruses followed by the plaque formation assay.

Plaque formation assay

A confluent monolayer culture of MDCK cells in a 6-well tissue culture plates was washed with serum-free MEM and then infected with 0.5 ml of influenza virus solution [500 pfu/ml = multiplicity of infection (MOI) of 2.5×10^{-4}] in serum-free MEM. After allowing 1 h at 37°C for virus adsorption, the cells were washed with serum-free MEM and then overlaid with MEM containing 0.8 % agarose, 0.2 % BSA and 1 $\mu\text{g}/\text{ml}$ L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma). After incubation at 37°C for 2–3 days, plaques were visualized by staining cells with 0.5 % amido black.

Results were represented as a ratio of the plaque number formed in the presence of EGb to that in the absence of EGb.

Hemagglutination assay

Influenza A/PR/8/34 virus (2×10^8 pfu/ml) was diluted nine times with phosphate buffered saline (PBS) (–) by twofold dilution each time, while 200 µg/ml of EGb was also diluted ten times with PBS (–) containing 0.2 % DMSO by twofold dilution each time. Fifty microliters of each diluted virus was mixed with 50 µl of each diluted EGb. These mixtures were then maintained at room temperature for 5 min. One hundred microliters of 0.5 % chicken erythrocyte suspension (Nippon Bio-Test Laboratories Inc., Japan) was added to each of these mixtures in 96-well round-bottom plates, and then the plate was incubated at room temperature for 30 min for hemagglutination. Results were represented as a plot where the *x*-axis and *y*-axis indicate concentrations of EGb and HA titer, respectively.

Statistical analysis

All of the data were represented as mean \pm standard error of the mean (SEM). Comparisons for all pairs were performed by Student's *t* test. A *p* value >0.05 was considered to be not significant. The calculations of 50 % cytotoxicity concentration (CC_{50}) and inhibitory concentrations with 50 % plaque reduction (IC_{50}) were performed by nonlinear regression using GraphPad Prism's "log (inhibitor) versus response – variable slope" function (GraphPad Prism Version 5.01 for Windows, GraphPad Software Inc.).

Results

Effect of EGb on the viability and growth of MDCK cells

Before examining the anti-influenza virus activity of EGb, we investigated whether EGb affects the viability and growth of MDCK cells, which are routinely used as host cells for influenza viruses. We evaluated the cell viability and growth by counting the number of living cells as a function of time using the neutral red assay as described in "Materials and methods". Cytotoxic effects of EGb were not observed at concentrations of <10 µg/ml ($CC_{50} = 180$ µg/ml) (Fig. 1a). Neither the growth rate nor the final cell density was affected by the presence of 10 µg/ml of EGb, whereas a marked decrease in the cell growth rate was observed at 100 µg/ml (Fig. 1b). Thus, EGb at a concentration of <10 µg/ml could be considered essentially

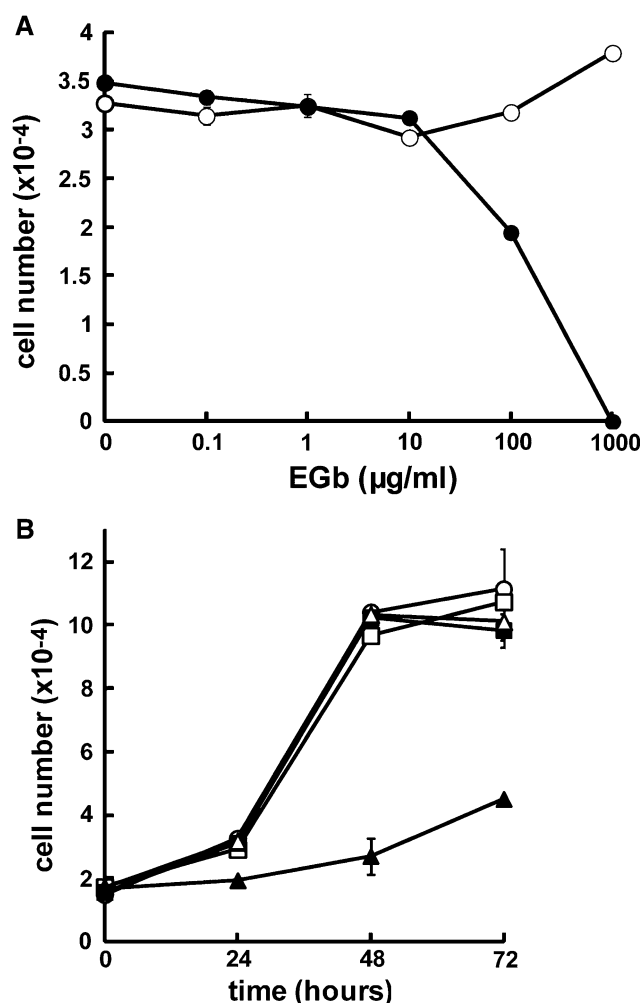


Fig. 1 Effect of EGb on the viability and the growth of MDCK cells. **a** MDCK cells (3.5×10^4) were seeded in 24-well tissue culture plates and incubated at 37 °C in the presence of various concentrations of EGb (closed circles) or solvent DMSO alone (open circles). After incubation for 24 h, the viable cell number was determined by the neutral red assay. **b** MDCK cells (2×10^4) were seeded in 24-well tissue culture plates and incubated at 37 °C in the absence (open circles) or presence of 10 µg/ml (closed square) and 100 µg/ml (closed triangles) of EGb, and 0.01 % (v/v) and 0.1 % (v/v) of DMSO alone (open square and open triangle, respectively). After incubation for the indicated periods, the viable cell number was determined by the neutral red assay

nontoxic to MDCK cells. We confirmed that the solvent DMSO had no effect on the viability and growth of MDCK cells in the range of concentrations used in this study (data not shown).

Inhibition of influenza virus infectivity by EGb

To examine whether EGb inhibits multiplication of influenza viruses, plaque assays were carried out as described in "Materials and methods". Cells were infected with influenza A/PR/8/34 virus at 37 °C for 1 h. The cells were

washed extensively with serum-free MEM and then overlaid with 0.8 % agarose in MEM containing EGb at various concentrations. The number of plaques and their sizes in the presence of EGb did not differ from those in the absence of EGb (Fig. 2a), indicating that EGb does not inhibit plaque formation by influenza virus infection. We further examined whether EGb is effective when mixed with viruses before exposure to cells. Influenza viruses were mixed with EGb at various concentrations at room temperature for 10 min and then exposed to MDCK cells. Under these conditions, EGb markedly inhibited viral infectivity in a dose-dependent manner (Fig. 2b). EGb at a concentration of 5 $\mu\text{g/ml}$ almost completely inhibited the plaque-forming activity. These findings suggest that EGb inhibits the initial step of influenza virus infection before the virus enters the cytoplasm. Next, we examined whether the inhibitory effect of EGb against influenza virus was direct or indirect. Plaque assays were performed using MDCK cells treated with EGb at various concentrations for 1 h before infection with the influenza viruses. The number and sizes of the plaques of the tested groups in the presence of EGb did not differ significantly from those of the control group in the absence of EGb (Fig. 3), suggesting that EGb

directly interacted with the influenza viruses and markedly reduced their infectivity.

Inhibition of hemagglutination by EGb

Influenza virus infection is initiated by the interaction of hemagglutinin (HA) on the virion with sialic acids on the host cell surface. To understand how EGb prevents virus adsorption to cells, we examined whether EGb competitively inhibits influenza virus-mediated hemagglutination. As shown in Fig. 4, EGb inhibited hemagglutination in a dose-dependent manner, suggesting that EGb interferes with the interaction between HA and sialic acids.

Susceptibility of other influenza virus strains to EGb

Our results suggest that EGb binds to HA and prevents virus adsorption to cells. We further examined whether the inhibitory effect of EGb is dependent on the type of influenza virus. EGb inhibited the infectivity of both influenza A/Udorn/72 (H3N2) and influenza B/Lee/40 viruses as well as of influenza A/PR/8/34 (H1N1) virus in an adsorption inhibition-dependent manner (compare Fig. 5a and b), albeit

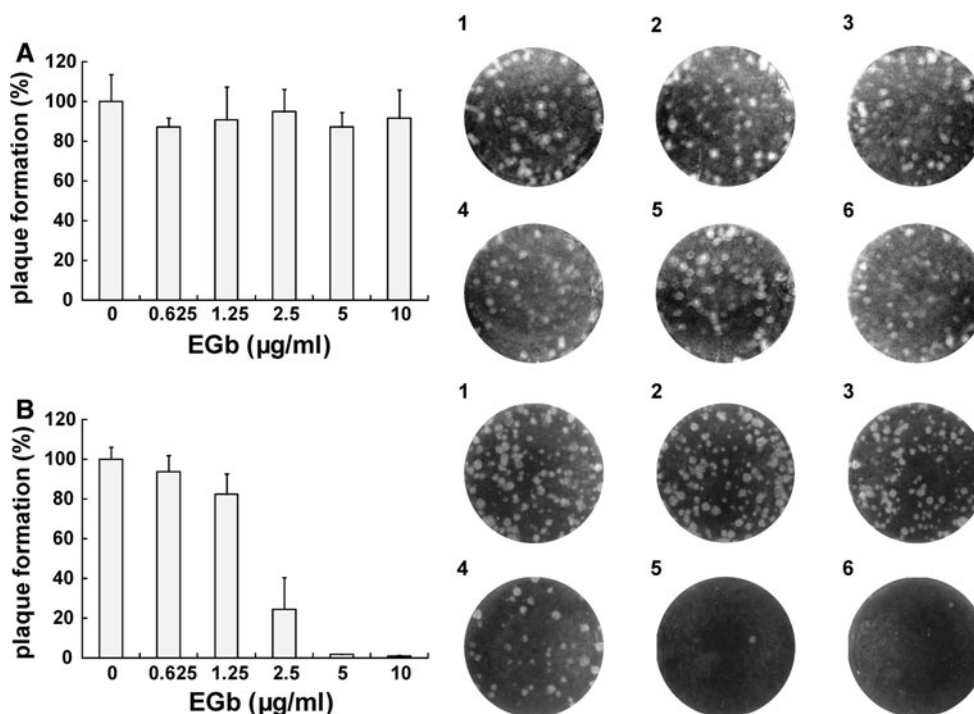


Fig. 2 Effect of EGb on plaque formation. Plaque assays were carried out as described in “Materials and methods”. **a** MDCK cells were infected with virus suspension (500 pfu/ml) and then overlaid with the overlay medium containing various concentrations of EGb. The profile of plaques is shown in the right panels. Panels 1, 2, 3, 4, 5 and 6 represent assays carried out in the presence of 0, 0.625, 1.25, 2.5, 5 and 10 $\mu\text{g/ml}$ of EGb, respectively. **b** Influenza A virus

(500 pfu/ml) was incubated with various concentrations of EGb prior to exposure to MDCK cells. The profile of plaques is shown in the right panels. Panels 1, 2, 3, 4, 5 and 6 represent assays in the presence of 0, 0.625, 1.25, 2.5, 5 and 10 $\mu\text{g/ml}$ of EGb, respectively. Results are represented as the percentage of the plaque number formed in the absence of EGb

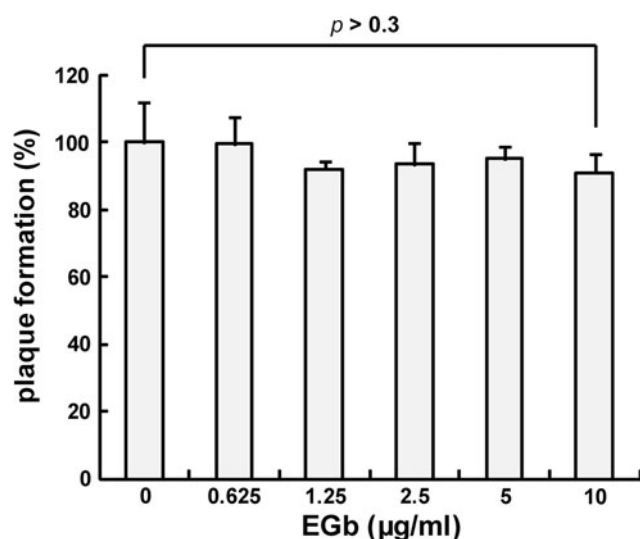


Fig. 3 Effect of pre-treatment of host cells with EGb on influenza virus infection. MDCK cells were exposed to EGb at various concentrations and incubated at 37 °C for 1 h prior to virus infections. After removing EGb, MDCK cells were inoculated with influenza A/PR/8/34 viruses (500 pfu/ml), and plaque formation assays were carried out as described in “Materials and methods”. Results are represented as the percentage of the plaque number formed in the absence of EGb. All data are represented as mean \pm SD, and the statistical analysis was performed using the *t* test to compare two groups

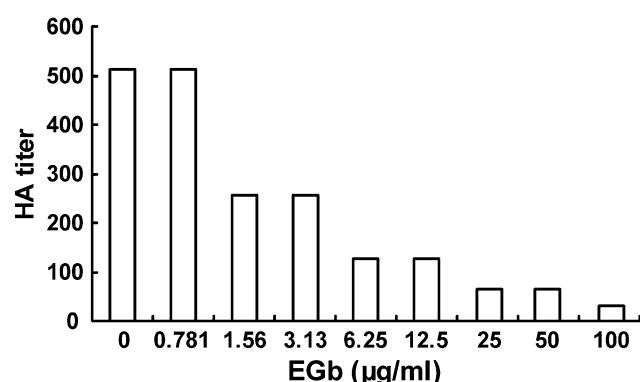


Fig. 4 HA titers of influenza A virus treated with various concentrations of EGb. Influenza A/PR/8/34 virus and EGb were diluted by twofold dilution each time and then mixed. After incubation at room temperature for 5 min, 0.5 % chicken erythrocyte suspension was added to each of these mixtures in a 96-well assay plate, and the plate was incubated at room temperature for 30 min for hemagglutination. Results are represented as a plot where the x-axis and y-axis indicate concentrations of EGb and HA titer, respectively. The result is representative of three independent experiments

with slightly different sensitivities. To confirm the difference in infectivity inhibition, the 50 % inhibitory concentration (IC₅₀) value of EGb was calculated for these three different strains of influenza viruses. Furthermore, the selectivity index (SI) was also calculated as the ratio of CC₅₀ to IC₅₀ (Table 1). The influenza A/PR/8/34 virus showed most

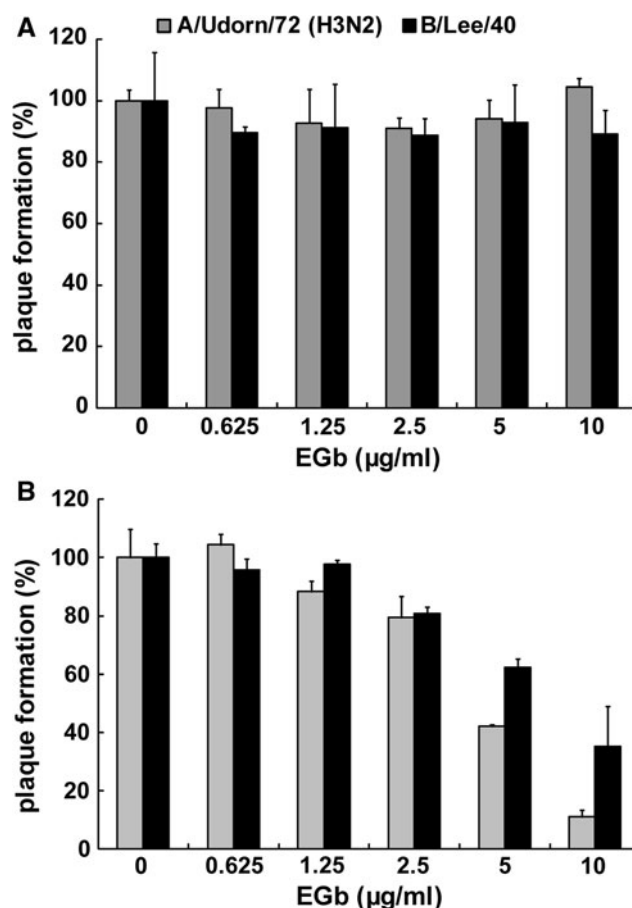


Fig. 5 Effect of EGb on plaque formation by two different subtypes of influenza virus. Plaque assays were carried out as described in “Materials and methods”. **a** MDCK cells were infected with 0.5 ml of 500 pfu/ml of influenza A/Udorn/72 (H3N2) and B/Lee/40 viruses and then overlaid with the overlay medium containing various concentrations of EGb. **b** Each influenza virus strain was diluted to 500 pfu/ml and incubated with various concentrations of EGb prior to exposure to MDCK cells. One hour after virus inoculation, MDCK cells were washed with serum-free MEM and subsequently overlaid with the overlay medium without EGb. Results are represented as the percentage of the plaque number formed in the absence of EGb. **a, b** Results of A/Udorn/72 (H3N2) and B/Lee/40 are represented by gray bars and black bars, respectively

sensitivity to EGb (Table 1). These findings suggest that the antiviral activity of EGb is not dependent on the type of influenza virus.

Discussion

In this study, we revealed the anti-influenza virus activity of *Ginkgo biloba* leaf extract (EGb). EGb acted directly on the influenza viruses and prevented their adsorption to the host cell surface, suggesting that EGb interfered with the interaction between the HA on the influenza virion and sialic acids on the host cell surface, although we could not

Table 1 Selectivity indices of EGb in three different influenza virus strains

Virus strain	IC ₅₀ (μg/ml) ^a	SI ^b
A/PR/8/34 (H1N1)	1.86	96.8
A/Udorn/72 (H3N2)	4.41	40.8
B/Lee/40	6.79	26.5

^a IC₅₀: 50 % inhibitory concentration of EGb was calculated from the results of the plaque formation assay performed as shown in Figs. 2b and 5b

^b SI: selectivity index was evaluated as the ratio of CC₅₀ to IC₅₀, i.e., $SI = CC_{50}/IC_{50}$

CC₅₀: 50 % cytotoxic concentration of EGb was calculated from the dose–response curve shown in Fig. 1a and its value (=180 μg/ml) was used for the calculation of each SI

All calculation was performed by using GraphPad Prism software as described in “Materials and methods”

exclude the possibility that EGb had a viricidal activity and directly inactivated the influenza virus.

The active constituents of EGb are standardized around the world; i.e., they contain 24 % flavonol glycosides (quercetin, kaempferol and isorhamnetin) and 6 % terpene lactones (ginkgolides and bilobalide). EGb also contains a class of condensed tannins, which are polymers composed primarily of flavan-3-ols (catechin and epicatechin) with a covalent bond between the individual flavonol units. Nakayama et al. [10] previously reported that two condensed tannins present in teas, (–)-epigallocatechin gallate (EGCG) and theaflavin digallate, bind to the HA of the influenza virus and inhibit its adsorption to MDCK cells. Furthermore, Song et al. [12] showed that catechin derivatives, including EGCG from green tea, inhibit not only the hemagglutination but also the NA activity of the influenza virus. The neuraminidase activity is thought to play a key role in the release of progeny virions from infected cells by cleavage of the sialic acid moieties of host cell receptors and in the prevention of self-aggregation of virions by cleavage of sialic acid still bound to the virus surface. These findings provide important insights into the molecular mechanisms of action of EGb.

Ginkgetin, a biflavone originally isolated from *Ginkgo biloba* leaves, has been found to inhibit the influenza virus sialidase [7]. However, our results showed that EGb prevented adsorption in the initial step of influenza virus infection. Therefore, in our study, a substance(s) in EGb other than ginkgetin may have been effective against influenza virus infection.

EGb was effective against the three different types of influenza viruses tested here, viz., the influenza A/PR/8/34 (H1N1), A/Udorn/72 (H3N2), and B/Lee/40 viruses, although the sensitivity towards EGb was slightly different among the three viruses. This finding suggests that EGb

may be a potential wide-range inhibitor against influenza virus infection.

When plaque assays were performed with overlay medium containing EGb, neither the number of plaques nor their sizes were affected (Fig. 2a). Since our results suggest that EGb acts directly on the influenza virus and prevents the initial step in viral infection, we expected that the infectivity of the progenitor virions would be decreased owing to interaction with EGb present in the overlay medium and, consequently, that the size of individual plaques would be reduced in the plaque assay. This discrepancy between the predicted and the experimental results may be explained by our recent findings: we disclosed a novel transmission mode for influenza viruses, the so-called cell-to-cell transmission mode [8]. Influenza viruses have generally been believed to be capable of spreading via *cell-free* virions released from infected cells depending on the activity of NA. However, in cell-to-cell transmission, progeny virions are retained on the infected cell surface even after budding and transmitted from infected cells to adjacent uninfected cells without being released into the outer environment. The cell-to-cell transmission of the influenza virus is dependent on functional HA but independent of NA activity. The present study may demonstrate that EGb cannot inhibit the cell-to-cell transmission of influenza viruses but is highly effective in decreasing the infectivity of *cell-free* virions. This suggestion is in line with the findings of a previous study in which higher concentrations of anti-HA antibody were needed for inhibition of infection through cell-to-cell transmission than for that through *cell-free* viruses [8].

The plaque assay using drug-containing agarose gels is one of the most reliable methods for detecting anti-influenza virus activity and is frequently used as a screening method. However, our findings raise concerns that a particular anti-influenza virus activity, such as the inhibitory effect found here in EGb, may have been largely overlooked in past studies.

In conclusion, we have shown that EGb interacts directly with influenza viruses and markedly reduces the infectivity of the viruses by preventing their adsorption to host cells. Furthermore, the inhibitory effect of EGb seemed not to be restricted to a certain subtype of influenza virus. Taken together, these findings indicate the usefulness of EGb as an antiviral agent for influenza, although further studies are necessary to confirm its anti-influenza virus activity *in vivo*.

In addition to the finding of the anti-influenza virus activity of EGb, we demonstrated an interesting and important insight(s) into the screening system for anti-influenza virus activity. As was the case for the anti-influenza virus activity of EGb found in this study, some candidates for antiviral agents may have been overlooked

in past studies because of the existence of the cell-to-cell transmission mode of influenza viruses. Therefore, our results signal a need for caution on the part of investigators trying to find anti-influenza virus compounds.

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